Comparative effects of Mono-Butyl Phthalate *in vitro* on testis explants from the fetal rat and human: comparison with effects of Di-Butyl Phthalate *in vivo* in the rat.

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Declaration

I have composed this thesis and the work described in this thesis is mine, unless otherwise credited. No part of this work is being submitted in support of another degree or qualification at this university or any other institute of learning.

Nina Hallmark, 2006

Acknowledgements

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Finally, to Frank, the biggest hug of all.

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Abstract

The incidence of disorders in human male reproductive health is increasing. It has been proposed this change is a direct consequence of environmental effects on growth and sex steroid induced alterations in programmed development. The aim of the experimental work described in this thesis was to investigate the effects of an ubiquitous environmental contaminant, the synthetic platiciser Di-butylphthalate (DBP) on the developing male reproductive tract. Any changes were compared to those symptoms of the human male clinical condition TDS (Testicular dysgenesis syndrome) which is increasing in incidence. To investigate whether DBP treatment induced any TDS-like changes, pregnant rats were gavaged daily with DBP at doses up to 500mg/kg/day from embryonic (e) day e13.5 up to e21.5. Morphological and hormonal parameters (testosterone, inhibin-B) were assessed in male rats aged e21.5 or adult ± *in utero* DBP exposure. Concurrently, untreated fetal rat and fetal human testis explants were cultured *in vitro* ± MBP (Mono butylphthalate), the DBP metabolite, at levels of up to 1mM, to investigate whether the array of adverse effects seen with the *in utero* exposure could be induced *in vitro*. Hormone production (testosterone, inhibin-B) and explant morphology were compared ± MBP exposure. The *in vitro* experiments were restricted to 48h exposure duration so an additional *in vivo* treatment regime was established to compare the endpoints induced after just 48h exposure *in utero*. Pregnant rats were dosed daily by gavage with 500mg DBP/kg/day on e19.5 and e20.5 only. Morphological and hormonal parameters (testosterone, inhibin-B) were assessed in male rats aged e21.5 ± 48h *in utero* DBP exposure. Findings were compared against those from the original *in utero* studies ± 8 days DBP exposure.

The long-term *in utero* exposure regime induced an array of changes in the phenotype of the male reproductive tract, evident in e21.5 and adult animals, including testis maldescent (cryptorchidism) and reduced fertility. Changes in testis morphology such as alteration in the distribution of Leydig cells and abnormal nucleation of gonocytes at e21.5 were also seen. The production of testosterone in testes at e21.5 was also significantly reduced following the DBP treatment, including a significant reduction in the protein expression levels of the steroidogenic enzyme P450scc. The *in vitro* experiments were unable to show a significant decrease in testosterone production after 48h exposure to 1mM MBP but when testosterone production was stimulated by hCG, the level of stimulation was significantly reduced when explants were co-incubated with hCG and 1mM MBP. The adverse effect of MBP on hCG stimulated testosterone production was seen in media collected from both fetal rat and fetal human testis explants after 48h co-incubation. The short-term *in utero* exposure regime induced a generally less severe array of changes in the phenotype and testis architecture of

the e21.5 male reproductive tract than those seen after 8-day *in utero* DBP exposure. However, a greater reduction in testis testosterone was seen than the long-term exposure induced, despite less of a reduction in the protein expression of P450scc.

The precise mechanism through which DBP induces its array of developmental abnormalities is still unclear but these studies support the hypothesis that even short term *in utero* exposure to DBP directly affects the developing testis, probably by acting on Leydig cells and disrupting normal testis endocrinology.

Abbreviations

Abbreviation	Definition
µg	microgram
/40w	week of gestation/ embryonic development in the human
17β-HSD	17 β-hydroxysteroid dehydrogenase
22-R-CHO	22-R-hydroxycholesterol
3β-HSD	3 β-hydroxysteroid dehydrogenase
ABC-HRP	avidin/biotin labelled horse-radish peroxidase enzyme conjugate
AMH	anti-Mullerian hormone (also known as Mullerian inhibiting substance)
ANOVA	Analysis of varianceα
AR	androgen receptor
ATP	adenine tri-phosphate
BrdU	5-Bromo-2'deoxyuridine-5'monophosphate
cAMP	cyclic adenine mono-phosphate
CE	coelomic epithelium
CIS	carcinoma-in-situ
CREB	cAMP response element binding protein
CSL	cranial suspensory ligament
d	postnatal day of age
DAB	3,3 DiAminoBenzidine
DBP	di-*n*-butylphthlate
DEHP	di-ethylhexyl phthalate
DES	diethylstilboestrol
Dhh	desert hedgehog
e	day of gestation/ embryonic development in the rat
e_m	day of gestation/ embryonic development in the mouse
ED	endocrine disrupter
EDS	ethane dimethane sulphonate
ELISA	enzyme linked immunosorbent assay
EPA	USA Environment Protection Agency
ER	endoplasmic reticulum
FSH	Follicle stimulating hormone
g	Gram (mass)
GCNA	Germ cell nuclear antigen
GI	Gastro-Intestinal (tract)
GnRH	gonadotrophin releasing hormone
GnRHa	gonadotrophin releasing hormone antagonist
h	hour
H&E	heamatoxylin and eosin
hCG	human chorionic gonadotrophin
HPG	Hypothalamic-Pituitary-Gonadal (axis)
Hpg	hypogonadal
Insl-3	insulin-like-factor-3
kDa	kilo daltons
kg	kilogram
KTZ	Ketoconazole
LH	Luteinising hormone

Abbreviation	Definition
M	Mole
MBP	mono butylphthlate
mg	milligram
min	minute
ml	millilitre
mM	millimole
MNG	multinucleated gonocytes
ng	nanogram
$P450_{C17}$	17 α-hydroxylase/ C17-20 lyase
$P450_{scc}$	cholesterol side-chain-cleavage
PAGE	PolyAcrylamide Gel Electrophoresis
PCB	polychlorinated biphenyl
PCG	primordial germ cells
PI	Proliferation Index
RIA	radioimmunoassay
s	second
S.D.	Standard deviation from the mean
S.E.	Standard error
S.E.M.	Standard error of the mean
Sf-1	Steroidogenic factor 1
SMA	smooth muscle actin
Sox-9	Sry related HMG box 9
SRB1	Scavenger receptor class B, type 1
Sry	Sex determining region on the Y chromosome
StAR	steroidogenic acute regulatory
TDS	testicular dysgenesis syndrome
TGF-β	transforming growth factor-beta
ts	tail somite
TUNEL	Terminal dUTP nick end labelling
Wt-1	Wilms tumour

Contents

1 Background

The incidence of human male reproductive health disorders appears to be increasing. The increased incidence of reproductive health disorders is potentially a serious public health concern, as reduced reproductive capability could significantly reduce the number of offspring and may ultimately render a species unsustainable. This extrapolation of the recent changes in human male reproductive health has catalysed an international push towards understanding more about male health issues. One scenario highlighted by the burgeoning research has been the observation that four clinical disorders, that encompass the majority of the adverse changes in male reproductive health, are hypothesised to share a common fetal origin (Skakkebaek et al., 2001). The disorders are collectively termed "testicular dysgenesis syndrome" (TDS) and are believed to be consequences of disordered development of the fetal testis. These manifest as:

- testis cancer
- low sperm count/ poor sperm quality
- cryptorchidism
- hypospadias

In order to explore whether environmental chemicals may be adversely influencing fetal testis development, the physiology of normal testis development will be examined. This will be compared against the phenotype described for TDS. Following review of reports on disrupted reproductive health in man and in wildlife, in the UK and abroad, a specific class of environmental chemicals was selected for further investigation as to whether or not such compounds could induce TDS in laboratory animals and whether they might do in humans. Comparisons will be made between data from novel studies presented here, involving acute exposure to high doses of a single chemical under laboratory conditions, both in vivo and in vitro, and published data regarding changes associated with continuous low-level environmental exposure conditions. The outcomes of these studies will be discussed in the context of contemporary findings. Together, this work should improve our understanding of the risk that ubiquitous synthetic chemicals pose to fetal testis development.

1.1 Testis formation and development

The development of the gonads generally governs the phenotypic sex of a mammalian fetus. As the gonadal anlage in the embryo can become male or female, sexual differentiation of the initial bi-potential gonad is a tightly regulated process. Formation of the male gonad, the testis, requires careful co-ordination of five processes: proliferation, migration and differentiation of the testis cell types as well their physical organisation into seminiferous cords alongside development of the testis specific vasculature. This section will review the

scientific understanding of testis development, up to the time when this project started, with occasional reference to the development of the female gonad, the ovary.

The gonads are unique in that they contain two distinct cell populations: somatic cells and germ cells. Each cell type has a distinct developmental origin: the somatic cells arise from the adjacent mesonephros and coelomic epithelium whereas the primordial germ cells migrate into the genital ridge from the yolk sac via the hindgut and dorsal mesentery (Figure 1.1). The migration of each major cell type into or within the testis and their functions will be reviewed.

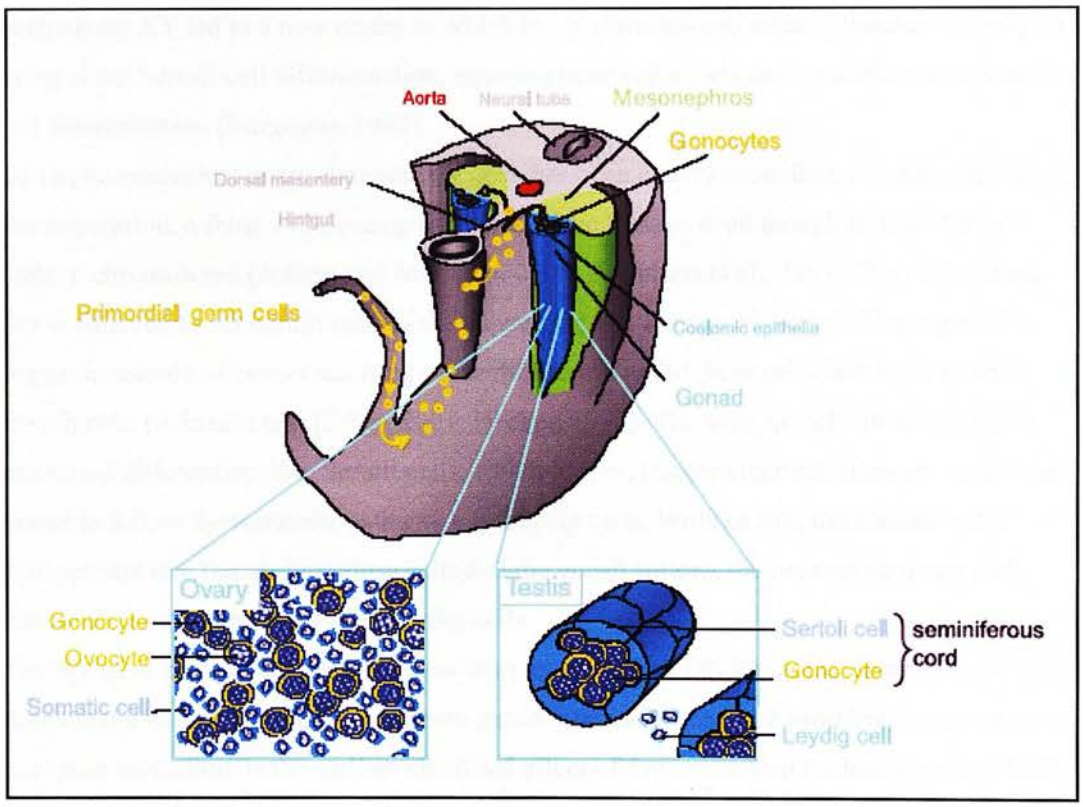

Figure 1.1 Posterior view of a rodent embryo showing gonadal development. In both sexes, the initial bi-potential gonad appears as a swelling of the mesonephros and coelomic epithelium. This region is colonised by primordial germ cells, where they differentiate into gonocytes. In the testis, the Sertoli cells surround the gonocytes forming basic seminiferous cords, interspersed with steroidogenic Leydig cells. Taken from: Rouiller-Fabre et al. 2003.

1.1.1 Sry

Testis development is dependent on the sex of the somatic cells in the developing gonad (Adams and McLaren, 2002). Sex is determined genetically by the presence of the Y chromosome for the mammalian male. Male sexual differentiation is confirmed by the hormonal secretions of the developing testis, as well as the development of the secondary sexual characteristics.

A pivotal revelation in reproductive biology emerged in the 1990's, with the identification of the gene termed "Sry" for "**S**ex determining **r**egion on the **Y** chromosome". This gene was identified as responsible for the commitment of an embryo to masculinisation, whether expressed in the gonad or via an ectopic transgene (Gubbay et al., 1990; Koopman et al., 1990). Prior to this point, it had long been assumed that the mammalian Y chromosome either encoded for or controlled the production of a diffusible testis-determining molecule, exposure to which was all that was required to divert the embryonic gonad along the testicular pathway. The finding that Sertoli cells in XX--XY chimeric mouse testes were exclusively XY led to a new model in which the Y chromosome acted cell-autonomously to bring about Sertoli-cell differentiation, equating mammalian sex determination with Sertoli-cell determination (Burgoyne, 1988).

So far, no equivalent ovary-determining gene has been clearly identified, but without normal Sry expression, a fetus will develop as a phenotypic female, even though it may carry the male Y chromosome (Adams and McLaren, 2002; Koopman et al., 1991). This is because Sry is believed to act within cells of the supporting cell lineage of the indifferent gonad to trigger a cascade of events resulting in the differentiation of these cells into male specific Sertoli cells (Albrecht and Eicher, 2001; Hacker, 1995). The somatic cells in which Sry is expressed differentiate into Sertoli cells, which in turn, trigger other cell lineages within the gonad to follow the testicular pathway e.g. Leydig cells. Without Sry, the somatic cells differentiate into female granulosa cells and the gonad follows the ovarian pathway with theca cells developing instead of Leydig cells.

The Sry gene product encodes a protein with an HMG box DNA binding domain, believed to regulate the transcription of downstream genes. The increase in cell proliferation in the male coelomic epithelium is the earliest identified effect of Sry expression (Schmahl et al., 2000). Expression of the Sry protein in the fetal mouse gonad 10.5 days after conception ($e_m10.5$) precedes the formation of testis cords, the earliest overt evidence of testis differentiation (observed by $e_m12.5$) and initiates male specific cell proliferation.

Comparisons of the predicted Sry protein sequences from different species have shown that apart from the HMG domain, regions are highly divergent even amongst closely related species. Comparison of human and mouse sequences is especially difficult since there is no region of extended homology apparent outside the HMG box (Hacker, 1995). This lack of homology makes it difficult to identify common regulatory regions and contributes to why no direct molecular target of Sry has yet been identified.

It should be noted that though Sry operates the "gender switch", and has provided a clear molecular anchor point for the study of the divergence of the male gonad from the

bipotential primordium, many other genes are required to complete sexual differentiation (Capel, 2000). The precise roles of some of the factors identified in mouse/rat sexual differentiation have not been precisely identified for human sexual differentiation (Hughes, 2001).

It may be worth noting that among farm animals, the pig presents with an intersex condition at a relatively high frequency i.e. XX sex-reversed individuals which are genetically female but with a true hermaphrodite or male phenotype. It has been clearly demonstrated that Sry is not involved in these phenotypes but genetic analyses suggest that instead, pig inter-sexuality is controlled via many genes (Pailhoux et al., 2001). Studies of XX sex reversal in the insectivorous mole (Talpa occidentalis) indicate that the intersex animals are functional fertile females and lack Sry but present with ovotestes. The trait is transmitted and maintained in the population. These results suggest that XX intersex moles may have arisen from a mutation of a gene located downstream from Sry in the testis determining pathway (Jimenez et al., 1993).

While expression of Sry may appear in other regions of the embryo or at other ages, this goes beyond the scope of this review (Capel, 2000; Hacker, 1995). A summary was published by Koopman (2001) following a symposium on the Genetics and Biology of Sex Determination listing the genes implicated in human and mouse sexual development, revealed by mutation analysis. The meeting emphasised that while a great deal of progress has been made in the years since Sry was first identified, most of the fundamental issues surrounding how its protein works are still unresolved (Koopman, 2001).

1.1.2 The mesonephros and coelomic epithelium

The gonads emerge around e10-11.5 in the mouse, on the ventro-medial surface of the mesonephros associated with a thickening of the coelomic epithelium in both sexes (Capel, 2000) (Figure 1.1). One of the first indications of masculinisation is the increase in the size of the male gonad by $e_m12.5$ (Schmahl et al., 2000). Several mechanisms could account for the size increase of the XY gonad following Sry expression but various studies show that XY gonads recruit cells from the mesonephros and coelomic epithelium (Albrecht et al., 2000; Karl and Capel, 1998; Merchant-Larios, 1998).

1.1.2.1 The mesonephros

Mesonephroi are derived from the embryonic mesoderm and are the precursor tissues for the gonads for both sexes (Tilmann and Capel, 2002). These transient organs form a vital part of the urogenital ridge, along with the pronephros (including the adrenal primordia) and the metanephros (the embryonic kidney precursor) (Sainio, 1997). The two mesonephroi evolveeither side of the gut mesentery and alongside the emerging gonad. Initially, both

sexes have mesonephric tubules that extend into the developing gonad towards the coelomic surface, in a process akin to branching morphogenesis in the developing kidney (Sainio, 1997).

As part of the reproductive tract, mesonephroi are subject to sex specific development. Both sexes have two ductal primordia in each mesonephros, though only one of the two ductal systems will normally develop, specific to the sex of the gonad and expression of supporting/ inhibiting factors. In the fetal female, the mesonephric duct regresses but the adjacent paramesonephric (Müllerian) duct persists and matures into the oviducts, uterus and upper part of the vagina of the adult female reproductive tract. In the fetal male, the Müllerian duct regresses due to the action of Anti-Müllerian Hormone (AMH) released by Sertoli cells. Additionally, in the fetal male the mesonephric (Wolffian) duct is actively maintained by the production of testicular testosterone (Capel, 2000; Jost et al., 1981). It matures into the epididymis, vas deferens and seminal vesicles of the adult male reproductive tract. Consequences of inappropriate testosterone or AMH production are detailed in a later section of this overview.

1.1.2.2 The coelomic epithelium

The coelomic epithelium is a single layer of cells derived from the mesoderm that lines the inside of the coelomic cavity, the precursor cavity that will form the pericardial, pleural and peritoneal cavities. The cavity is underlain by a basement membrane (coelomic mesothelium) that proliferates into the adjacent loose connective tissue, enabling coelomic epithelium cells to migrate towards the mesonephros, creating the gonadal anlage (Birk et al., 2000).

Karl et al (1998) were able to dye individual coelomic epithelium (CE) cells and follow their proliferation and migration into the interior of the gonad during early development, at tail somite stages (ts) 15-30 (e_m10.5-11.5). Labelled CE cells were observed to proliferate, then migrate into the parenchyma of the gonad and go on to become various somatic cell lineages. These cell lines both expressed (i.e. Sertoli cells) or didn't express (e.g. Leydig cells) the transcription factor *Wt-1* protein. Those CE cells that did not arrive in the gonad until after ts18 always remained outside the seminiferous cords becoming part of the interstitial cell population, whereas those that migrated during ts15-17 went on to become *Wt-1* expressing intratubular cells, i.e. Sertoli cells (Karl and Capel, 1998). Different studies have shown that mesonephric cells can differentiate into cells that have ultra-structural features akin to those of normal steroidogenic Leydig cells (Merchant-Larios, 1998).

By ts30, the movement of CE cells ceased and the basement membrane beneath the coelomic epithelium was no longer discontinuous but began to thicken and form the "tuniga

albugninea", a tough capsule that covers the entire testis (Karl and Capel, 1998). These data suggest that the coelomic epithelium is a dynamic cell population that is actively involved in morphogenesis of the gonad, including contributing cells to the somatic cell population. These findings corroborate previous observations that differentiation of Sertoli and Leydig cells proceeds in the absence of the mesonephros (Merchant-Larios et al., 1993).

1.1.2.2.1 The coelomic vessel

By $e_m12.5$, a large blood vessel can be seen on the developing testis, just under the coelomic epithelium. This arises from epitheliuml cells that migrate into the gonad from the mesonephros and endothelial cells. Together with the unisex vasculature already present in the $e_m11.5$ gonad, these endothelial cells form the coelomic vessel and a supporting vascular system specific to the male gonad. This sexual dimorphism suggests that vascularisation plays a role in defining the early architecture of the testis, including the rapid export of testosterone after $e_m13.5$ (Tilmann and Capel, 2002). It is hypothesised that vascularisation and seminiferous cord formation are simultaneous and mediated by the Sertoli cells but there is no supporting data at this time.

1.1.2.3 Migration and cord formation

In order to become a testis, the indifferent gonad needs to acquire the various cell lines that make up the male gonad and organise them into structured seminiferous cords. Germ cells have an established extra-embryonic origin (detailed in a later section) whereas the major somatic cell types arise from the embryonic urogenital tissues, i.e. the mesonephros and coelomic epithelium as already outlined. Additional testis cell lines include the peritubular myoid cells and various ill-defined interstitial cell types as well as as cells associated with the testis vasculature. When labelled mesonephroi were grafted to unlabeled gonads, endothelial and peritubular myoid-like cells migrated into the gonad. It has therefore been proposed that these cells might be the mesonephric cells required for seminiferous cord formation (Merchant-Larios et al., 1993).

Experiments have shown that mesonephric migration is Sry activated. Delayed Sry expression postpones migration and thus cord formation, even resulting in aberrant ovotestis formation (Albrecht et al., 2000; Capel et al., 1999). There is a critical timepoint after which normal testis cord formation cannot be initiated i.e. if the movement of precursor cells into the gonad has not started before $e_m12.5$ (rat e14.0), then cord formation is disrupted (Tilmann and Capel, 1999) (Figure 1.2). Normal migration starts before ts18 ($e_m11.5$) and has been suggested to last until $e_m16.5$ (rat e18.5), as evidenced with studies where gonads explanted at $e_m11.5$, without a mesonephros, show normal development, supporting the idea that migration must be initiated prior to this age. (Tilmann and Capel, 2002). Additionally,

there is no migration of cells between an XX mesonephros and its adjacent gonad or between an XY gonad with an adjacent XX mesonephros, but CE cells do contribute to the complement of cells that make up the female gonad (Tilmann and Capel, 1999).

Pre-Sertoli cells, together with migrating mesenchymal cells, form the seminiferous cords around the primordial germ cells that migrate in from the hindgut via the mesonephros. Some mesenchymal cells flatten out and differentiate into peritubular myoid cells to form the basal lamina with the Sertoli cells, enveloping the clustered germ cells into basic cords, while others remain in the interstitial spaces between the cords (Figure 1.2). The co-ordination of all these cells into a testis, with defined seminiferous cords and interstitium, is orchestrated by the only cell that expresses Sry, the Sertoli cell (Hacker, 1995). The Sertoli cell is the logical candidate to induce mesonephric migration via a paracrine factor, yet unidentified. Like Sry expression, cord formation starts in the central region of the gonad and spreads to the poles of the testis (Albrecht and Eicher, 2001).

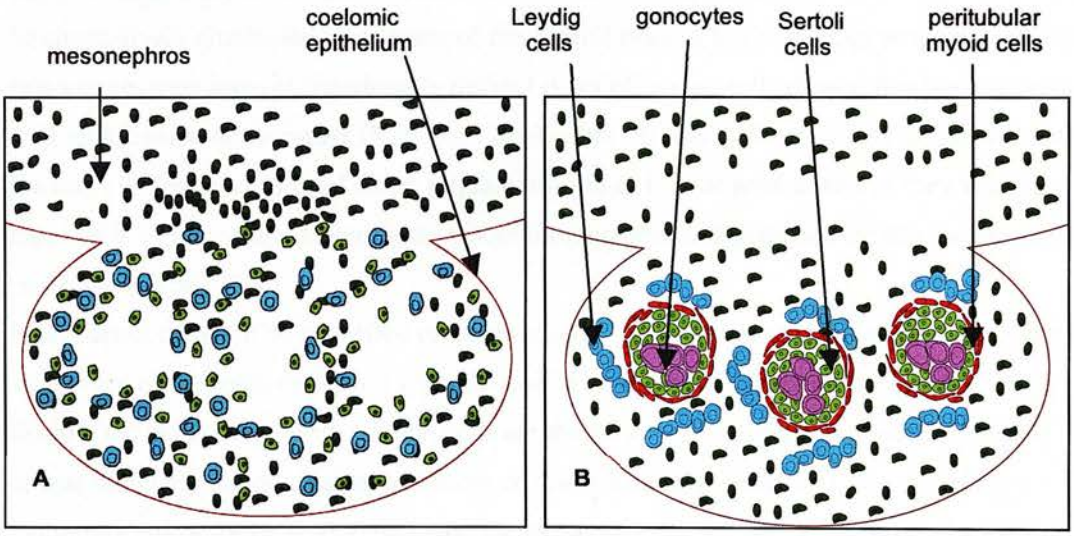

Figure 1.2: Cartoon of seminiferous cord formation in fetal life. Panel A represents the indifferent gonad with precursor somatic cells present. Panel B represents the formation of the seminiferous cords showing how the Sertoli cells (pale green), Leydig cells (blue) and peritubular myoid cells (red) organise themselves to form recognisable cords following influx of the primordial germ cells (purple). Adapted from movie clip developed for RM Sharpe, 2003.

Proliferation is also critical to gonad formation, as shown by the failure of gonad formation in mouse models in which the Lhx-9 gene has been knocked out. This gene is normally required for the growth of the gonads via the proliferation and invasion of the epithelium

into the mesenchyme (Birk et al., 2000). The role of proliferation and the emerging male gonad is returned to later in this review.

1.1.3 Sertoli cells

The cells of Sertoli are named after the Italian scientist Enrico Sertoli (1842-1910) who first described this 'special cell' in a paper published in 1865, long before the discovery of Sry (Skinner, 2005).

Sry is the principal initiator of the cascade of cellular and molecular events that enable the development of the testis, yet is only detected in the male urogenital ridge for a short period, just prior to when overt sexual differentiation is apparent. This transient pulse of Sry expression is detected in the pre-Sertoli cells of the developing genital ridge, from $e_m10.5$ to a peak around $e_m11.5$ after which it plummets until undetectable by $e_m12.5$ (Albrecht and Eicher, 2001; Hacker, 1995). Due to the expression of Sry exclusively in the Sertoli cells, these cells are thought to orchestrate male development by influencing the differentiation of other cell types in the XY gonad.

The sole source of pre-Sertoli cells is believed to be the CE, though the mesonephros cannot be conclusively eliminated as a source of pre-Sertoli cells. It has even been proposed that the two sources may provide functionally distinct types of Sertoli cells though this has not been well investigated in mammals (Karl and Capel, 1998). If this is the case, it is suggested that the non-CE Sertoli cells must either migrate prior to $e_m11.5$ or arise in situ as they were not reported in organ culture experiments where mesonephroi and gonads were re-assembled at this stage (Capel, 2000).

Fetal Sertoli cells can be identified in the developing testis as they lie alongside the basement membrane of the testis cords and appear cuboidal with almost spherical nuclei, each with a distinct nucleolus (Figure 1.3). Germ cells are the only other cell type in the cords but they appear distinctly larger, rounder and more centrally located (Figure 1.3). The Sertoli cell cytoplasm envelops the fetal germ cells. The presence of Sertoli cells can be confirmed by the detection of specific biomarkers, typically either the cytoplasmic glycoprotein AMH (Anti-Müllerian Hormone) or the transcription factor Wt-1 (Hacker, 1995; Sainio, 1997).

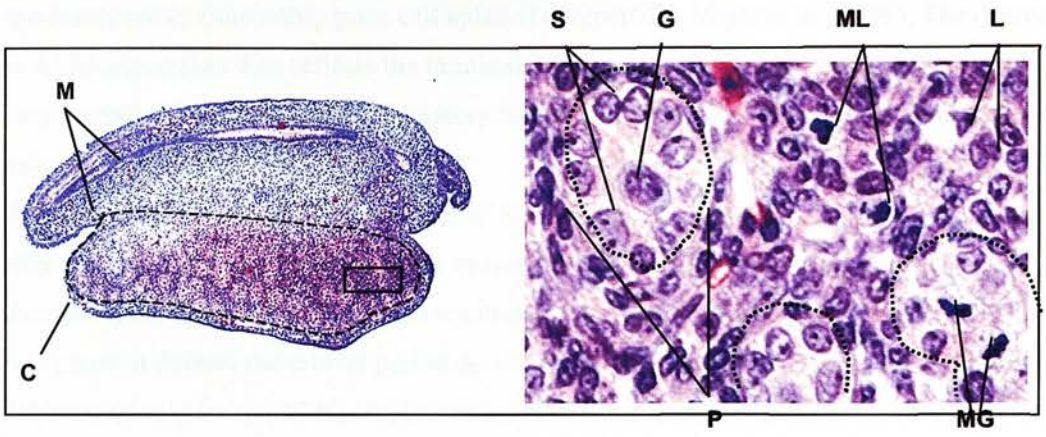

Figure 1.3 H&E stained section of e15.5 fetal rat genital ridge. The left panel shows a longitudinal section of a developing testis (---) surrounded by the coelomic epithelium (C) with the mesonephros (M) attached (x10 magnification). An area (rectangle) enlarged in the right panel, shows cord formation: Sertoli cells (S) surround gonocytes (G), including mitotic gonocytes (MG), bounded by the basal lamina (‴) of the seminiferous cords, which is in turn surrounded by peritubular myoid cells (P). Between the cords are the interstitial cells including probable Leydig cells (L), some probable mitotic Leydig cells (ML) and occasional un-defined interstitial cells (pink).

Many genes expressed in the fetal Sertoli cells are associated with testis development. Sry, the "testis determining gene" has already been mentioned. Some of the other genes encode for transcription factors such as Wt-1 or Sox-9 while others encode for secreted proteins such as AMH and inhibin-B. These four gene products are described in more detail below but this is definitely not an exhaustive list of genes critical to testis development. Others include Dmrt1, Wnt-4, Lhx-1, Emx-2, Lhx-9, Sf-1 and Dax-1 but to expand on the contribution made by all of these is beyond the scope of this review. An thorough reference book that details the functions of these genes and many more associated with the Sertoli cell, was just published (Skinner, 2005).

1.1.3.1 AMH

Following the determination phase of sex differentiation, re-enforcement and regulation of further testis development is influenced by hormone production and expression of the cognate receptors in target tissues (Hughes, 2001). One such hormone is Anti-Müllerian Hormone (AMH), strongly expressed in the immature Sertoli cell cytoplasm of the perinatal testis. It is also weakly expressed in ovarian granulosa cells from birth to the end of ovarian activity, menopause (Josso et al., 2001). In normal testes, the switch-off of AMH expression is associated with the appearance of primary spermatocytes, suggesting that their presence had an inhibitory effect on AMH, though AMH expression was also down-regulated in testes

lacking germ cells (because of anti-cancer treatment) or testes with a complete lack of spermatogenesis (idiopathic germ cell aplasia) (Rajpert-De Meyts et al., 1999). The decrease in AMH expression thus reflects the terminal differentiation of Sertoli cells and is probably only partially dependent upon a regulatory factor associated with the onset of meiosis in the adjacent germ cells (Sharpe et al., 2003).

Amh gene expression is an early marker of Sertoli cells. *Amh* expression begins 20 hours after the onset of Sry expression in the mouse testis, at a time when Sry transcripts are at their peak, around $e_m11.5$. While this result does not prove a direct interaction between the two genes, it defines the critical period during which SRY must act to initiate Sertoli cell differentiation (Hacker, 1995). Additionally, AMH was detected in culture media following incubation of $e_m11.5$ gonads without a mesonephros, indicating that some Sertoli cells must be present in the gonad by then (Merchant-Larios et al., 1993).

AMH is a member of the TGF-β superfamily of growth factors which includes another Sertoli cell product, inhibin. AMH is a homodimeric disulfide-linked glycoprotein with a molecular weight of 140kDa (Josso et al., 2001). The main role of AMH is to cause regression of the female Müllerian ducts, hence its alternative name – Müllerian Inhibiting Substance (MIS). This sex-specific event is carried out via binding to AMH type II receptors (AMHR-II) on mesenchymal cells which are proposed to induce apoptosis of the Müllerian epithelium via a poorly defined paracrine mechanism (Hughes, 2001). The identity of a type I receptor is controversial.

AMHR-II is also expressed on Leydig cells (Racine et al., 1998; Teixeira et al., 1999). AMHR-II-deficient mice exhibit Leydig cell hyperplasia suggesting AMH could be a negative modulator of Leydig cell differentiation and function, as aside from regression of the Müllerian ducts, AMH inhibits the postnatal differentiation of Leydig cell precursors into mature steroidogenic cells (Racine et al., 1998). In Leydig cell lines, AMH has been shown to reduce in vitro testosterone production to 10% of the basal level, due to the repression by AMH of the transcription of the gene for the steroidogenic enzyme $P450_{C17}$ (Teixeira et al., 1999).

Given that the expression level of AMH varies between fetal, neonatal, pubertal and adult testes as well the ovary, it is reasonable to assume that different mechanisms regulate AMH during development in males and females (Watanabe et al., 2000). In the fetal male, AMH expression is tightly regulated to ensure that it is expressed during the critical period of Müllerian duct regression. The absence of the hormones AMH and testosterone, produced by the developing male gonad, creates a permissive environment for the differentiation of the Müllerian ducts and female reproductive organs (Habert et al., 2001).

To facilitate this regulation, the AMH gene has several response elements within its promoter. Response elements have been identified for: the orphan nuclear receptor Sf-1; the high mobility group protein Sox-9 and the transcription factor GATA-4, all of which are integral to normal testis development (Josso et al., 2001). In the testis, AMH expression is initiated by Sox-9 and up-regulated by Sf-1 with interaction from GATA-4, Sox-9 and Wt-1 (Watanabe et al., 2000). Germ cells can also influence AMH expression. Germ cells that have initiated meiosis effect a repression of Sertoli cell AMH expression in the same seminiferous tubule. However, the precise molecular pathways through which regulation by these factors is imposed, or the factors influencing AMH expression in ovarian granulosa cells, remain unclear (Josso et al., 2001). Outside the testis, AMH might be associated with delayed airway branching, specifically within the developing male lungs (Catlin et al., 1997).

1.1.3.1.1 Sox-9

The human AMH proximal promoter can bind the transcription factor Sox-9 (Sry-related HMG box-9), a Sertoli cell factor closely associated with Sertoli cell differentiation and the initiation and regulation of AMH expression. Sox-9 can cooperate with Sf-1 in this activation process (De Santa Barbara et al., 1998; Wilhelm and Englert, 2002). Sox-9 is an High Mobility Group (HMG) protein transcription factor i.e. works by binding to the minor groove of DNA causing it to unwind and widen (Migeon, 2000). Sry is also an HMG protein. Possibly regulated by Sry, the Sox-9 gene is associated with the differentiation of Sertoli cells and the suppression of ovarian differentiation (Migeon, 2000). It is expressed at low levels in the genital ridge prior to Sry but is dramatically up-regulated following initiation of testis development. Sox-9 expression decreases in the fetal rat until postnatal d2 then at d15 Sox-9 protein strongly reappears in the testicular cords. In the adult rat, Sertoli cells are positive for Sox-9, whereas ovaries are negative for Sox-9, confirming its sex-specific nature. The prominent presence of Sox-9 in the developing testis and at puberty suggests that this protein is needed at important phases of aggregation and reorganization of the Sertoli cells. The age- and stage-specific presence of Sox-9 in the testicular cords and in the seminiferous tubules of the adult suggests that Sox-9 also may have a pivotal role in germ cell differentiation (Frojdman et al., 2000). Sox-9 is sufficient to induce testis formation in mice, indicating that it can substitute for the sex-determining gene Sry, but unlike Sry, Sox-9 expression is not transient (Vidal et al., 2001). Mutations in Sox-9 are associated with male-to-female sex reversal in humans and a severe dwarfism syndrome, campomelic dysplasia (Morais da Silva et al., 1996).

1.1.3.2 Wt-1

The Wilms' tumour gene (Wt-1) codes for a zinc finger transcription factor. Mutations of the gene result in embryonic tumours of the kidney, sometimes associated with gonadal dysgenesis such as in Denys-Drash syndrome (decreased Wt-1 binding to DNA) or Frasier syndrome (abnormal intron 9 splice variant). The 50bp Wt-1 gene has 10 exons and encodes a protein with four zinc finger DNA binding domains. Alternative splicing creates four isoforms with roles in transcription regulation as well as RNA processing, with 96% conservation between the human and mouse amino acid sequences (Moore, 1999). Splice variant II has an insertion between zinc fingers 3 and 4, the KTS sequence (+KTS isoform), and accounts for the majority of Wt-1 in a cell. The different splice variants have different roles in sexual development (Wilhelm and Englert, 2002). The +KTS variants are important regulators of Sry in the sex determination pathway compared to the -KTS forms, which are associated with the survival of the gonadal primordium (Hammes et al., 2001).
Cells that express Wt-1 protein are found in the urogenital ridge prior to the expression of Sry and sexual differentiation, indicating it is involved in the differentiation of many genitourinary tissues, all potential sites of Wilms' tumour as well as proliferation/ tumourogenesis (Hirose, 1999). Wt-1 can also activate target genes such as Dax-1. Though the role of Dax-1 in gonad formation is not firmly established, it is known that if it is duplicated, it induces XY sex reversal in humans whereas Dax-1 inactivation leads to reduced testis size and impaired spermatogenesis, (Wilhelm and Englert, 2002).
Inactivation of both Wt-1 alleles results in the failure of the kidneys and gonads to develop and the mice to die in utero around e_m12-14. These embryos also have fatally malformed hearts because of disrupted migration of Wt-1 expressing epicardal cells from the proepicardial organ to the myocardium surface. When these embryos were "rescued" and survived beyond mid-gestation, they were found to have impaired adrenal gland development. Wt-1 is not found in the developing adrenal cortex but is expressed earlier in development when the adrenal and gonad share a common primordium. This steroidogenic anlage also expresses steroidogenic factor -1 (Sf-1), found in the Leydig and Sertoli cells of the testis. Wt-1 (-KTS splice variant only) and Sf-1 interact to regulate the expression of AMH (Moore, 1999). Sf-1 is activated by Wt-1: the -KTS splice variant binds to and transactivates the Sf-1 promoter (Wilhelm and Englert, 2002). Wt-1 regulates many genes during urogenital development, though characterisation of this pivotal transcription factor and its associated co-factors is ongoing, as it is still not known precisely how this protein contributes to early gonad development. Wt-1 is continuously expressed in Sertoli cells in the fetal and the adult testis.

1.1.3.3 Inhibin

Inhibin is a member of the transforming growth factor (TGF)-β superfamily, members of which share structural similarities but are diverse in their biological activities. Inhibin is a heterodimeric glycoprotein made up from an α-subunit plus either of several β-type subunits (A, B or C in the human, plus E in the mouse, plus D in Xenopus), joined by disulphide bonds (Ying, 1988). Homodimers of the β-subunit are known to activate FSH secretion and are called "Activin". The inhibin specific α-subunit dimerises with any of the β-subunits, though in mammals, only the β_B (inhibin B) subunit is associated with male reproductive health (Illingworth et al., 1996).

Inhibin B is produced by the Sertoli cells as precursor α and β subunits which are believed to dimerise intra-cellularly into a 120kDa precursor protein, which undergoes maturational processing into a 60kDa intermediate form and finally a 30kDa mature form. The mechanism of inhibin signalling is still unclear but two receptors have recently been characterised in the rat testis: β-glycan (localised to the Leydig cells) and P_{120} (found on Leydig and Sertoli cells). In the human fetal testis, β-glycan was localised in the peritubular myoid cells and interstitium (fetal Leydig cells) but it is not assumed that these are the only targets for Sertoli cell derived inhibins (Anderson et al., 2002).

The expression pattern for the inhibin α-subunit depends on developmental age. First detected in rat testes at e14.5, immunostaining has revealed inhibin-α protein expression in Sertoli cells and interstitial cells. As pregnancy progresses the interstitial cell staining intensifies (Majdic et al., 1997a). By the day of birth, low levels are still expressed in the cytoplasm of Sertoli cells but interstitial staining intensity has lessened and by postnatal d5 all interstitial staining has vanished, leaving low-level Sertoli cell expression. The bioactive form i.e. the dimeric form of testicular inhibin, is not detected until after birth (Noguchi et al., 1997). The role of the bio-inactive inhibin-α expressed in the fetal Leydig cells is unclear.

Inhibins are hormones involved in the regulation of a number of diverse physiological functions e.g. regulation of FSH production (Majdic et al., 1997a). The only bioactive inhibin form in fetal and adult male rat and human circulation has the β_B subunit, abbreviated to inhibin-B, and is predominantly secreted by the Sertoli cells (Anderson et al., 2002). However, the mechanism by which inhibin-B exerts its biological effect is still under investigation (Meachem et al., 2001). Inhibin is most likely not bioactive during fetal testis development but vital to postnatal testis function. In the postnatal testis, the level of secreted inhibin-B is proportional to the number of Sertoli cells in a testis, as shown by the increase in

levels concurrent with normal and disrupted Sertoli cell proliferation in the postnatal rat testis (Sharpe et al., 1999). The final number of Sertoli cells in a testis has an important effect on the circulating levels of inhibin-B but not exclusive control, though the relative contribution of these factors requires further investigation. Inhibin levels can be affected by various factors, e.g. Sertoli cell maturation, route of excretion, change in germ cell complement or changes in the feedback sensitivity with FSH (Sharpe et al., 1999). During adult spermatogenesis, it is the germ cell population rather than FSH that has the greater influence on inhibin-B levels, though the inverse correlation between inhibin-B and FSH is still evident in healthy males (Meachem et al., 2001). At puberty, when Sertoli cell proliferation is complete and spermatogenesis commences, the basal adult inhibin-B production level is fixed and therefore is considered an index of Sertoli cell density (Meachem et al., 2001).The role of FSH is covered in more detail in a later section.

1.1.3.4 Proliferation

Following the formation of the seminiferous cords, the testis undergoes a dramatic sex specific growth spurt. Sertoli cell proliferation is vital, as their finite number will limit the number of germ cells that can be supported through spermatogenesis in the adult testis. Germ cells are completely dependent on the physical and metabolic support of the Sertoli cells to complete their development into spermatozoa (Orth, 1982; Sharpe et al., 1999). Sertoli cell mitosis essentially occurs in sexually immature testes in mammals (Jegou, 1992; Sharpe et al., 2003). In mammals there are probably three distinct phases of Sertoli cell proliferation: fetal, perinatal and prepubertal, all of which are potentially vulnerable to disruption, thus affecting the final Sertoli cell number (Sharpe et al., 2003). In the rat, Sertoli cells proliferate throughout fetal development until around 15 days after birth, producing the adult population of Sertoli cells prior to the completion of puberty (Orth 1982; 1984). Experiments with administration of recombinant FSH to neonatal rats saw an increase in final Sertoli cell number of 149% (Meachem et al., 1996). Conversely the suppression of FSH by the administration of a GnRH antagonist or Diethylstilboestrol, saw around a 50% decrease in the final number of Sertoli cells (Atanassova et al., 1999). Fetal rat Sertoli cells do not appear to respond to FSH but do express the FSH receptor after e18.5 (Rouiller-Fabre et al., 2003). Any reduction in Sertoli cell numbers such as decreased proliferation during the fetal/ neonatal period can result in a decrease in the maximum spermatid numbers produced by the mature adult, presenting as reduced sperm counts and possibly reduced fertility.

Differentiation

Around puberty, the Sertoli cell undergoes a dramatic change from an immature proliferative state to a terminally differentiated and non-proliferative, mature state. Tight junctions form

between adjacent Sertoli cells creating the blood: testis barrier. This provides a unique environmental niche for the germ cells and removes them from direct access to any non-Sertoli cell secreted products (Jegou, 1992; Sharpe et al., 2003).

The mature Sertoli cell extends its cytoplasm from the basement membrane of the seminiferous tubule to the lumen. Mature Sertoli cells carry out a wide range of functions including: structural support of the seminiferous epithelium, displacement of germ cells and release of sperm; formation of the Sertoli cell blood-testis barrier; secretion of tubule fluid (= lumen formation); nutrition of germ cells; phagocytosis of degenerating germ cells/ germ cell materials (Jegou 1992). Each Sertoli cell hosts a number of germ cells across a range of stages of differentiation into mature spermatozoa.

1.1.3.5 Endocrine influences on the Sertoli cell

The postnatal and adult testes are largely dependent on endocrine regulation *via* hormones such as the gonodotrophins (FSH and LH) to maintain testicular functions, but this system is not established until late in fetal life (Rouiller-Fabre et al., 2003; Sharpe et al., 1999). The regulation of the fetal testis, by contrast, is largely endocrine independent, implying that it is regulated via testis produced paracrine factors, such as AMH and testosterone. Testosterone is utilised by the fetal Sertoli cell to convert into oestrogen, via the P450 aromatase enzyme. Oestrogen is thought to suppress androgen production by immature Leydig cells, though its specific paracrine action is unclear. However, fetal Sertoli cells do not express the androgen receptor protein (AR). In the rat, AR expression is seen in the Sertoli cells from around postnatal d6, but not in the human until late puberty (Sharpe et al., 2003). This implies that any androgen influence on fetal Sertoli cells must be indirect. The roles of LH and FSH are discussed in more detail in section 1.2.

This review of the Sertoli cell is focused on the fetal phase of testis development so only skims over the role of the mature Sertoli cell and spermatogenesis. It should be noted that a fetal testis and its cells are not a mini version of the adult testis but has a unique pattern of protein expression (Rouiller-Fabre et al., 2003). However, it should not be underestimated how much influence the development of the immature Sertoli cell has on the successful function of the adult Sertoli cell and testis (Sharpe et al., 2003).

1.1.4 Peritubular myoid cells

These cells are poorly represented in the literature, despite their contribution to the architecture of the testis. They arise from the mesonephros and form the perimeter of the seminiferous cords in the testis, enveloping the immature Sertoli cells and gonocytes (Schmahl et al., 2000; Yao and Capel, 2002). It is proposed that a minimum number of myoid cells is required to establish and maintain initial testis cord formation following Sry

expression in Sertoli cells. Studies in which the mesonephros was separated from the gonad prior to the completion of mesonephric cell migration resulted in a reduced myoid cell population. This was proposed to reduce the production of matrix proteins that contribute to the formation of the basal lamina, between the Sertoli cells and the myoid cells, that separates the Sertoli cells from the stromal compartment of the testis, which in turn limited testis cord formation (Buehr, 1993; Merchant-Larios, 1998). Different studies showed that disruption of the signalling between the Sertoli cells and the myoid precursors (by Cyclopamine) or of mesonephric cell migration (by Forskolin) also resulted in disrupted testis cord formation (Yao and Capel, 2002).

Peritubular myoid cells proliferate during fetal life but this ceases shortly after birth, suggesting that peritubular myoid cell proliferation is not a major contributor to postnatal testis growth. At puberty, these cells change shape to become flatter and longer, increasing in volume rather than number (Palombi et al., 1992). Peritubular myoid cells also have a role in testicular development that is likely to be androgen regulated as they express androgen receptor protein, though this role requires to be clarified (Anderson et al., 2002). There is no ovarian cell type that is analogous to this testis cell type, though connective tissues may fulfil a similar role (Adams and McLaren, 2002).

1.1.5 Germ cells

The germline is responsible for the passing on of genetic information between generations. Primordial germ cells (PGC's) arise from the proximal epiblast of the embryo and their migration into the developing gonad, from the yolk sac via the gut mesentery, is one of the first events of gonad formation (Adams and McLaren, 2002). The germline is first detectable at $e_m7.25$ as a small population in the extraembryonic mesoderm near the base of the allantois (a membranous sac that grows from the lower gut in mammalian embryos). These migrate towards the urogenital ridge so that by e_m12, they are located in the gonadal anlage. During this migration, the PGC's are stimulated to initiate proliferation, completed by $e_m13.5$ (Richards, 1999). In the mouse embryo PGC numbers increase from less than one hundred to approximately four thousand during the period of their migration (Godin and Wylie, 1991). The regulation of this proliferative phase is thought be autonomously programmed into the PGC's, dependent on time rather than the number of cell doublings, as rare ectopic PGC's in the adrenal cortex stop mitotic proliferation in near synchrony with those in the ovary (Richards, 1999).

PGC's migrate in association with each other via intercellular cytoplasmic processes (filopodia). Following migration into the gonad, the PGC's round up and collaborate with somatic cells to form the primary sex cords (Bendel-Stenzel et al., 2000). Cadherins are a

family of intercellular adhesion molecules that are involved with the sorting of cells during morphogenesis, of which E-cadherin has been proposed to maintain the inter-PGC contacts in both sexes. Removal of E-cadherin disrupts the condensation of the germ cells in the genital ridge, resulting in an increase in the number of ectopic (extragonadal) PGC's (Bendel-Stenzel et al., 2000). Normally ectopic germ cells do not survive but the majority of paediatric germ cell tumours arise outside the gonads (Bendel-Stenzel et al., 2000).

Having migrated and proliferated, the male germ cells still need to differentiate into sperm. Already they have matured from PGC's into fetal germ cells (gonocytes) as seen by the declining expression of GCNA1 protein (Germ cell nuclear antigen-1). As this differentiation can also occur in vitro, it must be independent of the process of migration or exposure to the genital ridge. For clarity in this document, the post-migratory germ cells will be referred to as gonocytes.

Once in the gonad, the gonocytes have the potential to develop into ooctyes or pre-spermatogonia. This differentiation process is completely dependent on the somatic cells of their gonad host, rather than their own chromosomal sex (Richards, 1999). Little is known about the interactions between the somatic cells and gonocytes in the early testis but it has been shown that without the presence of male somatic cells, PGC's develop as if female (Adams and McLaren, 2002). Male gonocytes, enveloped by Sertoli cells, are mitotically quiescent until after birth whereas in female embryos, the pre-oogonia enter meiosis such that by birth they have reached the diplotene stage (Richards, 1999).

Gonocytes are not required by the male gonad to enable cord formation or differentiation of the Sertoli cell, but somatic cells are required by the male gonocytes to prevent them from becoming meiotic and direct them towards spermatogenesis. If male gonocytes are recovered from the mouse genital ridge prior to $e_m11.5$, they can revert to developing as oocytes. By $e_m12.5$ male gonocytes can synthesise prostaglandin D_2 and are committed to spermatogenesis, coincident with the onset of AMH expression by the adjacent Sertoli cells (Hacker, 1995). By $e_m13.5$ female gonocytes are committed to oogenesis, so if the gonocytes have not been exposed to a male environment prior to $e_m13.5$, they assume an oocytic fate. Regardless of their genetic sex, germ cells within a gonad will enter meiosis by $e_m13.5$ and follow normal ovarian differentiation unless there are signalled not to (Adams and McLaren, 2002).

It is proposed that the synthesis of prostaglandin D_2 by the recently differentiated male gonocytes promotes the differentiation of Sertoli cells in the gonad. As Sertoli cells direct testis formation, this paracrine factor will consolidate rather than regulate male sex determination (Adams and McLaren, 2002). The effect of the absence of prostaglandin D_2 on

testis development has been difficult to ascertain due to the multiple prostaglandin D_2 synthase enzymes and receptors. Mice generated with mutations in genes vital for prostaglandin biosynthesis (e.g. $Cox1^{-/-}$ or $Cox2^{-/-}$), die perinatally due to failure of the pups to successfully commit to pulmonary respiration as the lack of prostaglandins prevents the closure of the ductus arteriosus between the pulmonary artery and the aorta (Loftin et al., 2001). No defect in the testes of these animals was reported, is consistent with the rationale that paracrine induction of Sertoli cell differentiation might have only a small role in testis development (Adams and McLaren, 2002).

In the rat the male gonocytes remain mitotically quiescent until just after birth, when they start to migrate from the cord centre to the basement membrane and begin differentiation into pre-spermatogonia (Boulogne et al., 1999).

The proliferation of PGC's is independent of their somatic environment but the survival and differentiation of gonocytes is completely regulated by their somatic neighbours. Although regulation of male germ cell development is not fully understood, it is presumably controlled by an interaction of multiple gene expression and the regulation of signal molecules in the local testis environment, such as: c-kit receptor/ steel factor (SF), Dazla and retinoic acid (Olaso and Habert, 2000).

- Mutations on the loci that encode the tyrosine kinase receptor c-kit and its ligand SF, show adult sterility, with reduced germ cells evident in e_m9-10 gonads, implicating effects on PCG migration (SF= possible mitogenic factor) and proliferation or loss through increased apoptosis (SF = anti-apoptotic around e_m11) (de Miguel, 2002; Loveland and Schlatt, 1997)
- Dazl mutants are infertile, with reduced germ cells evident by e_m19.5 but not at e_m15. The Dazl gene encodes for an RNA binding protein associated with the survival of gonocytes during the period of quiescence in fetal life (Ruggiu et al., 2000)
- Retinoic acid (RA), the bioactive form of vitamin A, increases the rate of apoptosis in e_m14.5 but not e_m18.5 gonocytes in vitro yet decreases apoptosis of d3 germ cells (Livera et al., 2000)

Gonocyte proliferation resumes postnatally between d1-d4, but their actual number falls during this time from around 100 000 per testis to almost 50 000. This decrease is not accounted for by the start of gonocyte differentiation into spermatogonia but by apoptosis. The demise of gonocytes by apoptosis is characteristic of germ cell development in the testis. Its physiological significance is not clear but is considered to be the result of mistakes in DNA replication being removed, to protect the integrity of the germline (Boulogne et al., 1999). The incidence of gonocyte apoptosis is affected by age, with peak rates over e14-15.5 decreasing to 0% by e18.5 and resuming on d2 - a similar pattern to gonocyte proliferation (Table 1.1). This balance of proliferation and apoptosis is responsible for the final population of differentiated pre-spermatogonia available to undergo spermatogenesis and ultimately

become mature spermatozoa. It is suggested that the control of germ cell mitosis is directed by Sertoli cells, based on in vitro studies of gonocytes ± Sertoli cells. Purified gonocytes only resumed proliferation when co-cultured with Sertoli cells otherwise they perished within 48h (Li et al., 1997). Postnatal germ cell survival is regulated by FSH, LH and androgens, but this is not evident in the fetal testis (Boulogne et al., 1999; Tapanainen et al., 1993).

Species (gestation length in days)	Stage of Germ cell differentiation (day of gestation, postnatal (d))						
	First detection of PGC's	Arrival of PGC's in genital ridge	Testis formation	Germ cell proliferation period	Mitotic arrest	Mitotic resumption	Gonocyte degeneration
Human (270)	21-28	35-42	42-45	70-140	(unclear)	puberty	0-5 years
Mouse (19)	7.5	11.5	12.5	13.5-16.5	16.5	d1	13.5-17.5, d7
Rat (21)	8	13.5	13.5	13-18	18	d3-4	14.5-17.5, d3

Table 1.1 Outline of the stages of germ cell differentiation during fetal and neonatal life in the rat, the mouse and the human (Olaso and Habert, 2000).

1.1.6 Leydig cells

The interstitial glands of Leydig in the testis are named after the German scientist Franz Leydig (1821-1908) who first described this cell in a manuscript published in 1850. These cells are not to be confused with the Leydig cells of the amphibian epidermis that are also named after him. Though Leydig's paper described the consistent appearance of cellular clusters between the seminiferous tubules in many species, the role of these cells did not become apparent until after he retired in 1887. In 1896, another German scientist, Friederich Reinke (1862-1919) proposed that

> "...these interstitial cells, produce an unknown product, which is transported through the lymph to the blood, ... and [has] something to do with spermatogenesis and probably with sex drive."

However, the endocrine role of the Leydig cells was not highlighted until a publication in 1903 by Bouin and Ancel. Pol Bouin (1870-1962) also developed an original chemical mixture to successfully preserve testes for histological analysis (reported in 1897, still used today and known as Bouin's fixative). During his employment at the University of Nancy, France, Bouin met Paul Ancel (1873-1961). Together they furthered the evidence that

Leydig cells produced a product that controlled male sexual characteristics. Subsequent investigators extended their work, such that during the 1930's this product was identified as a steroid and became known as testosterone, and during the 1950-60's it was demonstrated that Leydig cells were the main source of testicular androgens in many mammals, including man and rodents (Payne, 1996; Saez, 1994).

Today, it is well established that a major role of Leydig cells is the production of testosterone and other endocrine hormones. This part of this review will focus on the biosynthesis of testicular testosterone, as it is critical for male sexual differentiation and the development of secondary male characteristics in addition to the initiation and maintenance of spermatogenesis, as well as the origin, proliferation and differentiation of the Leydig cell.

1.1.6.1 Steroidogenic cell origin

There are two types of testicular Leydig cells, fetal and adult, which develop as distinct cell lineages (Mendis-Handagama and Ariyaratne, 2001). It has been suggested that due to the simultaneous occurrence of the fetal Leydig cells and the adult population of Leydig cells, and their distinct formation, that these cells lines are distinct and developmentally unrelated (Kerr and Knell, 1988). The first population develops during fetal life, downstream of Sry, but regresses following masculinisation of the male urogenital system, although a few fetal type Leydig cells do persist into adulthood (Kerr and Knell, 1988). The second population appears postnatally (Habert et al., 2001). Though the details of the origin and differentiation of these two lineages has not been clearly determined, they share a common function - the production of testosterone, for the support of masculinisation and spermatogenesis respectively.

Adult Leydig cells are proposed to derive from adult peritubular mesenchymal cells whereas fetal Leydig cell origin is less clear. They are known to migrate into the gonadal anlage from the mesonephros, though this may not be their exclusive origin (see 1.1.6.1.1) (Habert et al., 2001; Siril Ariyaratne et al., 2000; Yao et al., 2002). In the rat, adult Leydig cells begin their differentiation during postnatal week two, via proliferation of non-steroidogenic precursor cells that differentiate and mature into steroidogenic adult Leydig cells through a series of morphological and functional changes. This process is dependent on the co-ordination of various cytokines (Meinhardt et al., 1996; Mendis-Handagama and Ariyaratne, 2001). The differentiation of adult Leydig cells occurs postnatally so the details of this process and the gene expression behind it extend beyond this review of fetal testis development. Some of the gene products associated with the origin of fetal Leydig cells, their development and function, are described in more detail below but this is not a complete or exhaustive list. A

reference book that outlines the functions of various genes, including those associated with the adult Leydig cell, is available (Payne, 1996).

1.1.6.1.1 Fetal Leydig cells

The initial embryonic origin of Leydig stem cells is still unclear but by observing development patterns using mesonephros/ gonad cocultures, it has been demonstrated that fetal Leydig cells are derived from the population of cells that migrate from the embryonic mesonephros (Capel, 2000; Habert et al., 2001; Merchant-Larios, 1998). Proliferation studies and Dil lineage tracing studies have revealed that cells from the coelomic epithelium also contribute to the population of interstitial cells of the developing testis though the fate of these is not clearly defined (Yao et al., 2002). Another proposed source of fetal Leydig cells is the embryonic neural crest due to their expression of neural proteins such as NCAM (neural cell adhesion molecule), suggesting a role for these molecules in the development and function of these steroidogenic cells (Mayerhofer et al., 1996). By e_m11.5, the interstitial cell precursors are already in the testis and by e_m12.5 (e15.5 in the rat) testosterone is being produced (Buehr, 1993; Merchant-Larios et al., 1993; Merchant-Larios, 1998; Yao et al., 2002).

It is uncertain what the key factors are, that regulate fetal androgen production, given that the initial differentiation of Leydig cells and androgen production is gonadotrophin independent as serum LH is not detected until later in gestation, around e17.5 in the rat (El-Gehani et al., 1998b; O'Shaughnessy et al., 1998). This observation is supported by studies in hypogonadal (hpg) mice in which almost no GnRH is present to stimulate LH production/ release by the fetal pituitary, yet normal fetal development of the male reproductive tract still occurs (O'Shaughnessy et al., 1998). During the perinatal period, endogenous gonadotrophins (e.g. LH) become essential for normal Leydig cell function. This suggests that in late gestation, both gonadotrophin support and gonadotrophin-independent support of Leydig cell function may exist. Perhaps these two systems synchronise to maximise androgen production during the androgen dependent virilisation of the male urogenital tract. What induces the late gestational conversion of steroidogenesis to gonadotrophin dependence is not clear, but may be related to birth itself, to changes in Leydig cell function as the fetal population starts to be replaced by the adult population, or to changes in Sertoli cell function as the germ cells start to differentiate (O'Shaughnessy et al., 1998).

Following their differentiation, the fetal Leydig precursor cells proliferate such that there are approximately $25x10^3$ cells by e17.5, reaching 90 $x10^3$ cells by e21.5. After birth, fetal Leydig cell numbers regress but are variable with a mean of 45-60 $x10^3$ per testis up to postnatal d100 (Kerr and Knell, 1988). As Sertoli cells differentiate ahead of the Leydig cells

(prior to e12.5 and after e12.5, respectively), it has been considered that Sry expression in the Sertoli cells induces a paracrine signal to a population of undifferentiated mesonephric cells to follow the Leydig cell lineage, though what this signal might be is also unclear (Habert et al., 2001). The Sertoli cell product AMH has been implicated in the inhibition of adult Leydig cell development and steroidogenesis (via $P450_{C17}$) but this is still being investigated, and there is evidence that AMH effects fetal Leydig cell development (Fynn-Thompson et al., 2003).

In the human, the first fetal Leydig precursors are identifiable during week 8 of gestation, following differentiation of the gonad during week 6, though testicular testosterone has been reported at week 6-7. This initial androgen production precedes gonadotrophin detection (during week 10) including any stimulation by placental chorionic gonadotrophin (hCG). This analogue of pituitary LH is produced maternally and is utilised later in gestation to stimulate androgen production coincident with virilisation of the male reproductive tract. Following birth, a second wave of Leydig cells develops, peaking around postnatal month 3, but overall the postnatal Leydig cell population decreases steadily until a nadir around 1 year after birth. Numbers are then steady until the pubertal wave of Leydig cell development. As for the rat, differentiation of the Leydig cells is recognised by their increase in cytoplasmic volume, appearance of the smooth endoplasmic reticulum and increased numbers of steroid-type mitochondria (reviewed in Habert et al, 2001).

1.1.6.1.2 Sf-1

The mesonephros and coelomic epithelium of the embryonic rat both include a population of cells that can be immunostained for Steroidogenic factor-1 (Sf-1, also known as Ad4BP or FTZF1 or NR5A1), a member of the nuclear hormone receptor family of transcription factors (Achermann et al., 2002; Birk et al., 2000). These cells can be tracked during embryogenesis and while some end up in the testis, others inhabit the developing adrenal gland, hence they are termed the "adreno-genital primordium" (Hatano et al., 1996). Sf-1 is expressed in a wide range of steroidogenic and non-steroidogenic tissues where it orchestrates the development of the reproductive axis (e.g. via the pituitary and hypothalamus) and adrenal glands (Hatano et al., 1994). More than 20 genes have been identified as influenced by Sf-1 via Sf-1 binding sites that regulate the transcription of the genes e.g. P450 steroidogenic enzymes, such as testicular $P450_{scc}$ and $P450_{C17}$ and the α-subunit of glycoprotein hormones (Achermann et al., 2002; Majdic et al., 1997b). The role these steroidogenic enzymes play in the synthesis of testosterone is detailed later in this review.

Immunoexpression studies have shown that Sf-1 is expressed in both the pre-Sertoli and the interstitial cells, especially the pre-Leydig cells of the presumptive testis. Examination of mice lacking Sf-1 revealed abnormalities of the genital ridge prior to the appearance of overt gonads. As testis cord formation progresses, Sf-1 levels fall in the Sertoli cells but rise in the fetal Leydig cells (Hatano et al., 1994).

The role of Sf-1 in the fetal Sertoli cell extends beyond the regulation of P450 steroidogenic genes (e.g. $P450_{aromatase}$) to other genes such as for the glycoprotein AMH. Sertoli cell specific Wt-1 binds to and transactivates the Sf-1 promoter, increasing Sf-1 expression, and together they up-regulate AMH expression, with interaction from GATA-4 and Sox9 (Watanabe et al., 2000; Wilhelm and Englert, 2002). This synergistic increase in AMH levels can be disrupted by the transcription factor Dax1 via direct interaction with the DNA bound Sf-1 protein (Tremblay and Viger, 2001). The expression profile of AMH is similar to that of Sf-1, even in ovarian granluosa cells (Hatano et al., 1994). Of the many genes whose products are known to be associated with the development of gonads post differentiation, both Wt-1 and Sf-1 are essential to gonadogenesis prior to differentiation, though the details of their hierarchy is still unclear (Birk et al., 2000; Wilhelm and Englert, 2002).

Targeted disruption of the Sf-1 gene was examined in mice. All Sf-1 null animals lacked adrenal glands and gonads because of apoptosis of the primordial organs just as both adrenal and gonad differentiation takes place. This supports adrenocortical insufficiency as the probable cause of death by postnatal d8. Male and female Sf-1 knockout mice had female internal genitalia, despite complete gonadal agenesis (Luo et al., 1994).

Sf-1 expression levels in the adrenal glands during fetal and postnatal development is not sex-specific, unlike for the testis. Sf-1 mRNA expression was first detected in the adrenal and the gonadal ridge of both sexes of fetal rats from e13.5, but after e14.5, the testis had a much greater level of Sf-1 expression than an ovary of the same age (Majdic et al., 1997b). This change in the Sf-1 expression levels in the gonads after sexual differentiation, indicates that maintenance of high level Sf-1 expression is sexually dimorphic, possibly a downstream target of Sry in the male. This is supported by the observation that both Sry and Sf-1 are expressed in the identical lineage of the somatic cells in the genital ridge (Hatano et al., 1994). The level of Sf-1 in the fetal testis peaks at e16.5 and is maintained until a dramatic decrease from postnatal d21 until only low levels are detected during adulthood. Conversely, fetal ovarian expression levels of Sf-1 increase postnatally from d7 until d21 then decrease to adult levels. For both sexes, Sf-1 levels fluctuate in the gonads during the first 3 weeks of postnatal life but not in the adrenals, confirming tissue specific and sex specific differential regulation of Sf-1 expression (Hatano et al., 1994).

An example of the tissue specific differential regulation of Sf-1 expression is the effect of mutations of Lhx9, a transcription factor and member of the LIM homeobox domain family. When Lhx9$^{-/+}$ mice were paired, the Lhx9 $^{-/-}$ progeny were infertile at 3 months old and had female internal genitalia, despite complete gonadal agenesis, including those that were genetically male, as for the Sf-1 null mice described above (Birk et al., 2000; Luo et al., 1994). At e_m11.5, the mutated Lhx9 gene inhibited normal proliferation of the gonadal ridge, particularly of the Sf-1 stained cells. Markedly reduced levels of Sf-1 expression were observed in the Lhx9 $^{-/-}$ gonadal ridge but not in the adrenal bud, indicating that Lhx9 might lie upstream of Sf-1 in the development cascade. By e_m13, the Lhx9 $^{-/-}$ gonads had regressed completely but the absence of the adrenals was not noted. The specific effect of the Lhx9 null mutation on gonad development, despite its normal widespread expression, was proposed to be a consequence of either redundancy of this gene in other Sf-1 expressing tissues (e.g. adrenal gland) or compensation by similar genes e.g. Lhx2 (Birk et al., 2000).

1.1.6.1.3 Dhh

Desert Hedgehog (Dhh) is a signalling protein essential for the early differentiation and proliferation of the Leydig cell lineage. Sertoli cells express the Dhh protein, whereas its receptor (Patched 1, Ptch1), is expressed by interstitial cells. Dhh is expressed downstream of Sry, and is proposed to be a positive regulator of the differentiation of steroid producing cells as it up-regulates the expression of Sf-1 in Ptch1 expressing cells outside the testis cords, specifying the Leydig cell lineage (Yao et al., 2002). It is likely that additional factors combine with Dhh to confirm the fate of the Leydig cell lineage from the precursor cells that contribute to the interstitium in the fetal testis.

1.1.6.1.4 PDGF-α

The platelet-derived growth factor (PDGF) family of ligands and receptors has a diverse range of roles across various physiological processes including migration, proliferation and differentiation, including early testis development(Brennan et al., 2003). There are two receptors (α and β) and four ligands (A, B, C and D) that dimerise and activate various intracellular pathways. Examination of *Pdgf-β*$^{-/-}$ mice showed no overt changes in XX or XY gonads but *Pdgf-α and Pdgf-*A null mice showed a sexually dimorphic expression pattern that was followed up in *Pdgf-α*$^{-/-}$ mice (Brennan et al., 2003). The role of PDGF signalling in the embryonic testis has not been thoroughly investigated but initial studies with *Pdgf-α*$^{-/-}$ XY gonads, revealed impaired cord formation, proliferation and endothelial cell migration. Additionally, *Pdgf-α* is associated with the differentiation of the fetal Leydig cell lineage as fetal Leydig cells are reduced or even absent as in Dhh$^{-/-}$ mutants (Brennan et al., 2003).

1.1.6.2 Cholesterol transport

Testosterone, a C_{21} four-ringed steroid, is produced following the enzymatic conversion of cholesterol (Figure 1.4). However, this is dependent on there being a source of cholesterol, i.e. dependent on the translocation of cholesterol, into the Leydig cell cytoplasm and then into mitochondria to start steroid biosynthesis. Cholesterol is a lipid molecule with 27 carbon atoms (C_{27}), a hydroxyl head region and a hydrocarbon tail region, separated by a rigid four-ringed steroid structure. It is found nestling among the phospholipid molecules that make up the lipid bi-layers of eukaryotic cell membranes (Alberts, 1994). This transport of cholesterol to the first enzyme complex in the steroid biosynthesis cascade is considered the rate-limiting step of steroidogenesis and, in Leydig cells this is facilitated by proteins SRB1 and StAR.

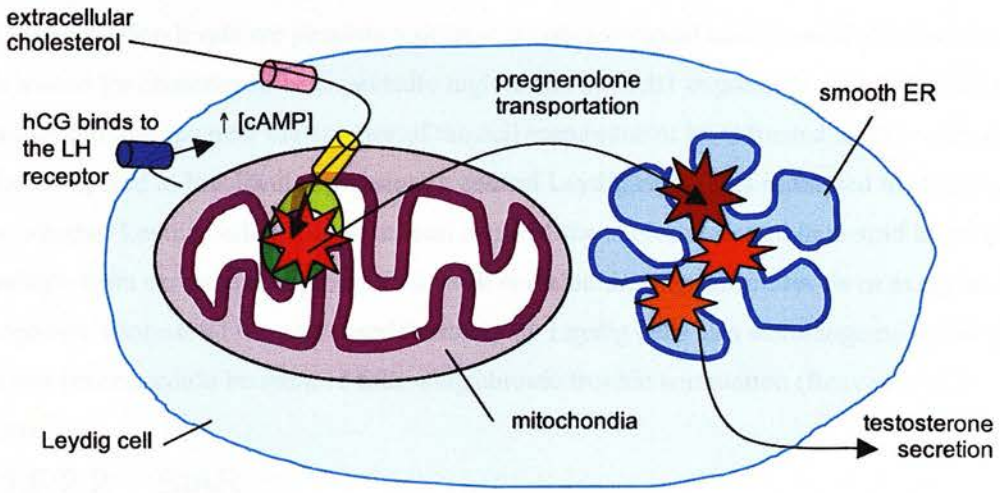

Figure 1.4: Simplified illustration of testosterone biosynthesis in a rat Leydig cell. Extracellular cholesterol is recruited into the cell cytoplasm (turquoise) via SRB1 (pink). Trophic stimulation of the LH receptor (dark blue) induces an increase in cAMP levels, which in turn mediates a multitude of intracellular activities, such as an increase in the mobilisation of intracellular cholesterol through the mitochondria membranes by StAR (yellow) where $P450_{scc}$ (red) converts it to pregnenolone. P450scc is found on the matrix (white) side of the inner mitochondrial membrane (purple) where it is associated with two proteins: adrenodoxin (pale green) and adrenodoxin reductase (darker green). Pregnenolone is transported to the smooth endoplasmic reticulum (ER) (blue) where it is converted to progesterone (via 3β-HSD, brown), to androstenedione (via $P450_{C17}$, orange) then to testosterone (via 17β-HSD, gold), which is mostly secreted out of the Leydig cell.

1.1.6.2.1 SRB1

Leydig cells differ from other cells in that they do not depend on exogenous lipoprotein-cholesterol sources during periods of normal steroid hormone production but utilise

endogenous cholesterol sources, such as plasma lipoproteins in the form of cholesteryl esters or oil droplets (Rao et al., 2003). They can be regulated by different factors than the similar steroidogenic cells of the adrenal glands (Payne and Youngblood, 1995).

Scavenger receptor class B, type 1 (SRB1) is an isoform of a high-density lipoprotein (HDL) receptor protein. SRB1 binds various lipoproteins, particularly HDL, then internalises only the cholesteryl ester particles it may carry, leaving the HDL carrier outside the cell (Figure 1.4). This way it mediated intracellular delivery of cholesterol from the HDL. There is a variant, type II, and though it exists in high levels in rodent testes, it appears to have no role in mediating intracellular cholesterol levels (Reaven et al., 2000). This mechanism is seen in small amounts through experiments with ^{3}H-labelled HDL-cholesteryl-esters and rat Leydig cells (Reaven et al., 2000). Once internalised, the cholesteryl esters may be stored as lipid droplets until needed, such as to be hydrolysed to produce free cholesterol for steroidogenesis (Rao et al., 2003).

SRB1 expression levels are possibly regulated by physiological conditions that reflect the cell's need for cholesterol. Unexpectedly high levels of SRB1 expression were observed by electron microscopy near the exterior of the cell membrane of hCG treated adult rat Leydig cells, compared to low level expression in control Leydig cells. This prompted investigation into whether Leydig cells acquired at least some of the cholesterol used in steroid hormone synthesis from exogenous sources. This work revealed that significant levels of exogenous lipoprotein cholesterol were not used routinely by Leydig cells as a steroidogenic precursor, but this process could be induced following chronic trophic stimulation (Reaven et al., 2000).

1.1.6.2.2 StAR

The transcription of the gene encoding the steroidogenic acute regulatory (StAR) protein is acutely regulated by trophic hormones via a cAMP second messenger pathway. In turn, StAR regulates the rate-limiting step of steroidogenesis in the adrenal cortex and the gonads i.e. the translocation of cholesterol from the cytoplasm of Leydig cells to the $P450_{scc}$ enzyme on the inner mitochondrial membrane (Figure 1.4). Without StAR to facilitate the journey, such low levels of hydrophobic cholesterol would cross the aqueous space between the two mitochondrial membranes that the observed physiological levels of steroidogenesis could not be supported (Stocco, 2001).

The gene encoding StAR is carefully regulated to ensure its proper temporal and spatial expression in steroidogenic tissues during development, and it is rapidly inducible in response to acute cAMP stimulation (Reinhart et al., 1999b). The molecular mechanisms underlying this regulation remain unclear. Functional analysis has demonstrated that

transactivation of the StAR promoter is Sf-1 dependent and immunostaining has shown that the expression of StAR correlates both temporally and spatially with Sf-1 expression (Pollack et al., 1997; Reinhart et al., 1999a). As StAR is not detected in the urogenital ridge of Sf-1 null mice, Sf-1 is proposed to developmentally regulate StAR expression. Dax1 is a negative regulator of StAR. Its exact mechanism is unclear but it works either by interacting with the StAR promoter sequence directly or via direct binding to and inhibiting Sf-1 regulated steroidogenesis (Stocco, 2001).

The StAR gene product is a precursor protein that undergoes two modifications from the 37kDa precursor to a 32kDa intermediate followed by cleavage into a mature 30kDa protein. This processing occurs during import of the precursor StAR protein from the Leydig cell cytosol into the outer then the inner mitochondrial membrane. This has been proposed to create contact points between the two membranes thus enabling cholesterol to be presented to $P450_{scc}$ for conversion to pregnenolone. On reaching the inner membrane, StAR is processed to its 30kDa form and the contact point is lost, making further cholesterol transfer dependent on de novo synthesis of the 37kDa protein. The levels of 30kDa StAR build up and do not display the anticipated turnover kinetics consistent with the loss of steroidogenic capacity (Stocco and Sodeman, 1991). It is not yet clear whether StAR can act as a cholesterol shuttling protein or affects cholesterol transfer less directly. The peripheral benzodiazepine receptor (PBR) is also located in the outer mitochondrial membrane and is associated with cholesterol transfer in mitochondria of steroidogenic cells, but it is not clear whether these proteins act together to promote cholesterol transfer (Papadopoulos et al., 1991; Stocco, 2001). The means by which the StAR gene is regulated and the exact mechanism of its action are still under investigation (Stocco, 2001). Studies have shown that StAR mRNA levels can be regulated by non-cAMP dependent factors such as upregulation via retinoic acid and thyroid hormone but down regulation by lipopolysaccharides, TGF-β, interferon-γ and TNF-α (reviewed in Habert et al 2001).

In the human, StAR protein has been immunolocated in fetal and adult gonads and adrenal glands, with intense staining in the Leydig cells of the fetal testis between 14-19w/40w, during peak fetal testosterone production (Pollack et al., 1997). Additional staining was noted in cell types that did not express the steroidogenic enzyme $P450_{scc}$ (e.g. Sertoli cells), suggesting that StAR has roles in metabolic processes other than pregnenolone biosynthesis. It was not detected in placenta suggesting existence of a StAR independent pregnenolone biosynthesis mechanism (Pollack et al., 1997). Mutations in the StAR gene result in the human condition "lipoid congenital adrenal hyperplasia" (lipoid CAH). Patients with this potentially fatal syndrome are unable to synthesise steroids as required from birth, despite

excessive levels of cholesterol and cholesteryl esters in their adrenal and testicular steroidogenic cells. This phenotype has also been noted in StAR null mice, confirming that the StAR protein has an indispensable role in normal steroidogenesis (Stocco, 2002).

1.1.6.3 Steroidogenesis

The main function of Leydig cells is the biosynthesis of the androgen testosterone. Androgens are vital during perinatal development to ensure completion of testicular descent, regulation of Sertoli cell number and initiation of spermatogenesis (Siril Ariyaratne et al., 2000). Testosterone is produced in response to acute and/or chronic stimulation, initiated by the pituitary hormone, luteinising hormone (LH), binding to specific high affinity LH-receptors on the surface of Leydig cells (Payne and Youngblood, 1995). The cellular protein kinase A (PKA) -dependent steroidogenic response is primarily mediated by phosphorylation of cyclic AMP (cAMP) -dependent transcriptional activators (e.g. Sf-1), resulting in the upregulation of StAR and other steroidogenic genes (Rao et al., 2003). More information on the role of pituitary hormones is detailed in section 1.1.8.

The conversion of cholesterol to testosterone in the testis requires the transcription of the genes that encode four catalytic proteins:

- cholesterol side-chain-cleavage ($P450_{scc}$)
- 3 β-hydroxysteroid dehydrogenase (3β-HSD)
- 17 α-hydroxylase/ C17-20 lyase ($_{P450C17}$)
- 17 β-hydroxysteroid dehydrogenase (17β-HSD)

These enzymes form an organised sequence, creating a steroidogenic cascade (Figure 1.5), yet are physically separated into different compartments within the Leydig cell (Figure 1.4) and subject to different mechanisms of regulation. Further details are outlined in the following sections. The steroidogenic enzymes fall into two major classes: the P450 heme-containing proteins and the hydroxysteroid dehydrogenases (Table 1.3).

Perturbations in any of the steps in the steroidogenic cascade (Figure 1.4), from the levels of binding of LH to its receptor, expression levels of the catalytic proteins, alterations in the mitochondrial or smooth ER membranes, could result in disrupted steroidogenesis and levels of testosterone production (Zirkin and Chen, 2000). Testosterone is the substrate for at least two enzymes: $P450_{aromatase}$ catalyses the aromatisation of testosterone into oestradiol (E_2) and 5α-reductase catalyses production of dihydrotestosterone (DHT), a more potent androgen than its precursor and vital for masculinisation of external genitalia (Habert et al., 2001).

Protein class/ name	Gene (Chromosomal location)		Catalytic conversion	Protein mass (kDa)
	Human	Mouse		
Cytochrome P450 's				
$P450_{scc}$	CYP11A1 (15)	Cyp11a1 (9)	cholesterol to pregnenolone	56
$P450_{C17}$	CYP17 (10)	Cyp17 (19)	progesterone to androstenedione	57
$P450_{arom}$	CYP19 (15)	Cyp19 (3)	testosterone to oestradiol	58
Hydroxysteroid dehydrogenases				
3β-HSD	HSD3β3 (1)	Hsd3β1 (3)	pregnenolone to progesterone	42-44
17β-HSD	HSD17β3 (9)	Hsd17β3 (13)	androstenedione to testosterone	34

Table 1.3 Summary of the major enzymes involved in testicular testosterone biogenesis expressed in human and mouse Leydig cells. Adapted from Hanukoglu (1992).

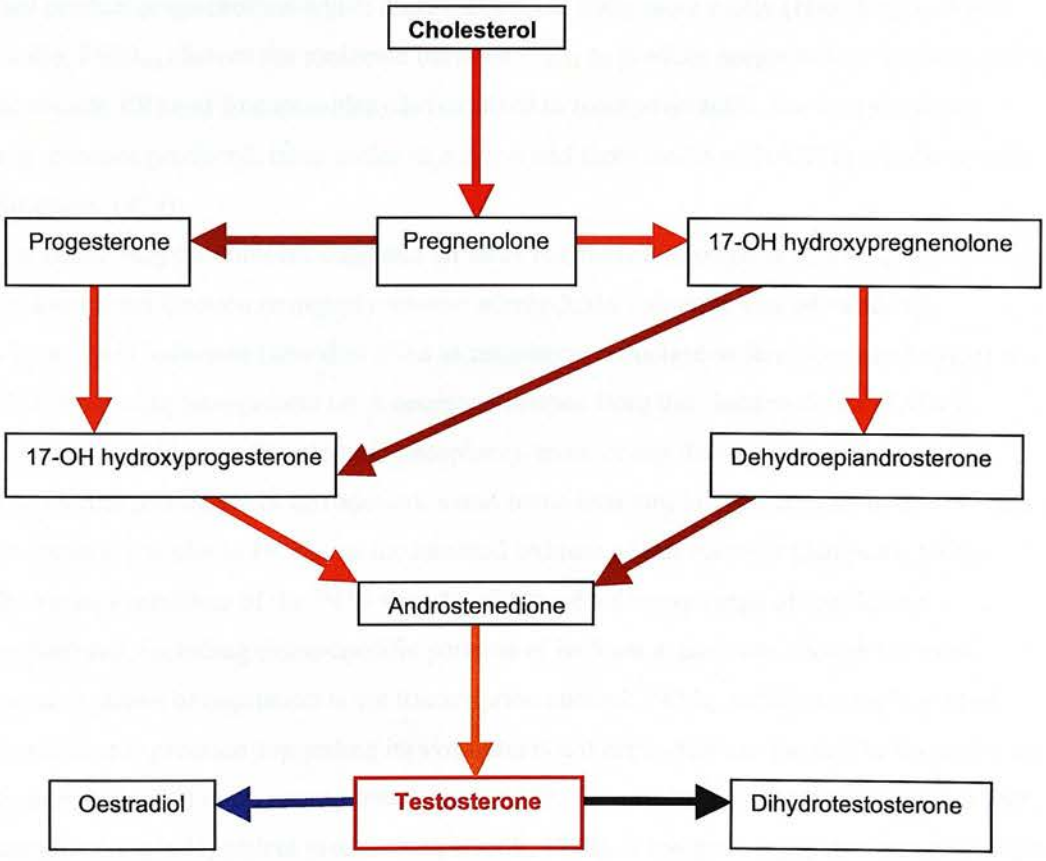

Figure 1.5: Simplified testosterone biosynthesis in a mammalian Leydig cell. One enzyme can catalyse more than one reaction and the most favourable route can vary between species, according to reaction kinetics. Each enzyme involved is represented by a different coloured arrow: red = **P450scc**, brown = **3β-HSD**, orange = **P450 c17**, gold = **17β-HSD**, blue = **P450 aromatase**, black = **5-reductase**.

1.1.6.3.1 P450scc

Cytochrome P450 cholesterol side-chain-cleavage enzyme ($P450_{scc}$ also known as 20, 22-desmolase) is a member of the membrane bound cytochrome P450 superfamily. $P450_{scc}$ is bound to the mitochondrial inner-membrane of steroidogenic cells, where it catalyses the conversion of the steroid precursor cholesterol to the metabolic intermediate pregnenolone (Pelletier, 2001). This is the rate-limiting enzymatic step in the biosynthetic cascade of all steroid hormones (Figure 1.5). The conversion of the C_{27} steroid cholesterol to the C_{21} steroid pregnenolone has three steps: first hydroxylation (therefore requires oxygen) at C_{22}, generating the intermediate 22-R-hydroxycholesterol and second, hydroxylation at C_{20} generating the intermediate 20,22-R-dihydroxycholesterol (Burstein, 1976; Burstein, 1975). These intermediates bind very tightly to $P450_{scc}$ without significant dissociation, unlike the final product pregnenolone which dissociates up to 600x more easily (Hanukoglu, 1992). Finally, $P450_{scc}$ cleaves the molecule between $C_{20\text{-}22}$ to produce pregnenolone (transported to the smooth ER) and isocaproaldehyde (oxidised to isocaproic acid). For every mole of pregnenolone produced, three moles of oxygen and three moles of NADPH are also needed (Simpson, 1979).

This single enzyme complex catalyses all three reactions at a single active site, facilitated by two associated electron transport proteins: adrenodoxin reductase and adrenodoxin. Adrenodoxin reductase (also described as testodoxin reductase or ferrodoxin reductase) is a FAD-containing flavoprotein i.e. it accepts electrons from the electron donor NADPH (nicotinamide adenine dinucleotide phosphate), transferring them to adrenodoxin (also described as testodoxin or ferrodoxin), a non-heme iron-sulphur protein that in turn mediates the electron transfer to $P450_{scc}$ as the terminal oxidase of this reaction (Simpson, 1979).

The various members of the P450 superfamily share a diverse range of regulatory mechanisms, including tissue-specific patterns of isoform expression, though the most common means of regulation is via transcription control. $P450_{scc}$ exhibits a high level of constitutive expression suggesting its synthesis is not dependent on, but can be increased by, stimulation by LH or its second messenger cAMP. Thus $P450_{scc}$ is regulated by both cAMP dependent and independent mechanisms (Oonk, 1990). It has been suggested that each P450 gene has its own cAMP response element (CRE) and even its own set of CRE binding proteins (Porter, 1991). Sf-1 has been shown to be necessary for the expression of $P450_{scc}$ as it binds to a consensus element in its promoter sequence.

Quantification of $P450_{scc}$ expression in human fetal testis samples (13-26/40weeks of gestation) showed that $P450_{scc}$ mRNA was most abundant in the adrenal gland, followed by the testis, placenta, and ovary and that none was detected in kidney, liver, spleen, intestine,

or muscle samples. The $P450_{scc}$ mRNA reached its peak between 14-16 /40w and showed significantly lower levels by 26/40w (Voutilainen and Miller, 1986). In the rodent, $P450_{scc}$ mRNA is detected in the mouse urogenital ridge from $e_m10.5$ and in the differentiated testis from $e_m12.5$ throughout gestation with little if any expression in the fetal ovary (Payne and Youngblood, 1995).

1.1.6.3.2 3β-HSD

Three beta-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) is found in the smooth endoplasmic reticulum/ mitochondria of the Leydig cell, where it catalyses multiple steps in the steroidogenic cascade (Pelletier, 2001). Overall, 3β-HSD converts 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids (Hanukoglu, 1992). In the Leydig cell, it converts pregnenolone to progesterone but it can also convert 17-OH hydroxypregnenolone to 17-OH hydroxyprogesterone or dehydroepiandrosterone (DHEA) to androsterone (Figure 1.5). The first reaction is the conversion of the 3β-equatorial hydroxysteroid (e.g. pregnenolone) via the coenzyme NAD+ to produce a Δ^5-3-keto intermediate (e.g. pregn-5-ene-3, 20-dione) and reduced NADH which activates the isomerisation of the Δ^5-3-ketosteroid to a Δ^4-3-ketosteroid (e.g. progesterone). All this happens without release of the intermediate or the NAD(H) cofactor (Payne, 1997).

In the rat, mouse and the human multiple isoforms of this protein have been identified, each with different tissue specific expression and catalytic kinetics for the same substrates. At least six isoforms have been described for the mouse, of which types I and VI are expressed in the testis (Abbaszade, 1997). In the human testis, only 3β-HSD type II is expressed but in the rat both types I and II are seen (Hanukoglu, 1992; Mason et al., 1993).

3β-HSD type I and 3β-HSD type VI are expressed in the mouse testis. When each isoform was measured individually, it was clear that the type I isoform was expressed at all ages from e_m13 to adulthood. In contrast, the type VI isoform was only expressed at significant levels during fetal life on e_m13 and then not again until after d10 postnatally (Baker et al., 1999). This finding is consistent with the observation in man that neonatally 3β-HSD is non-detectable until puberty (Mack et al., 2000).

The distribution of 3β-HSD among fetal tissues is widespread and not restricted to the male or the reproductive tract. In the rodent, 3β-HSD expression parallels that of $P450_{scc}$ and its mRNA is detected in the mouse differentiated testis from $e_m12.5$ then throughout gestation with little if any expression in the ovary until after birth (Payne and Youngblood, 1995). 3β-HSD exhibits a high level of constitutive expression in Leydig cells, however, in vitro studies demonstrated that LH and cAMP treatments increased the level of 3β-HSD

transcription after 24h, perhaps suggesting that like $P450_{scc}$, 3β-HSD is regulated by both cAMP dependent and independent mechanisms (Keeney and Mason, 1992). However, endogenously produced testosterone, but not oestradiol, represses cAMP-induced 3β-HSD mRNA synthesis (Majdic et al., 1997b). Meanwhile, levels of testicular mRNA encoding 3β-HSD type I were similar in normal and hpg mice (i.e. gonadotrophin deficient model), suggesting that the expression of this protein was independent of trophic stimulation (O'Shaughnessy et al., 1998).

The expression of 3β-HSD could be dependent on the expression of Sf-1 as for the P450 enzymes, though this is possibly exclusive to adrenal 3β-HSD expression (Payne and Youngblood, 1995).

1.1.6.3.3 P450C17

Cytochrome P450 17 alpha hydroxylase/ C_{17-20} lyase ($P450_{C17}$ also known as $P450_{17\alpha}$) is a member of the membrane bound cytochrome P450 superfamily. $P450_{C17}$ is found in the smooth endoplasmic reticulum of the Leydig cell where it catalyses the conversion of progesterone into androstenedione (Pelletier, 2001). The conversion of the C_{21} steroids to C_{19} steroids has two steps: first 17α-hydroxylation, also required for cortisol synthesis, then cleavage of a two-carbon side chain at C_{17} - C_{20} producing a C_{19} steroid and acetaldehyde (Hanukoglu, 1992). Human $P450_{C17}$ can also perform 16α-hydroxylation, perhaps depending on the orientation of the steroid at the time of hydroxylation (Swart et al., 1993). The absence of $P450_{C17}$ from the rat adrenal means that corticosterone (derived via progesterone) rather than cortisol (derived via 17α-hydroxyprogesterone) is the major glucocorticoid product. Additionally, the absence of $P450_{C21}$ in Leydig cells channels the steroid biosynthetic pathway towards androgen production ($P450_{C21}$ catalysis determines the biosynthesis of adrenal steroid hormones from Δ^4 steroids) (Hanukoglu, 1992).

$P450_{C17}$ catalyses both reactions at a single active site, without the 17α-substrate leaving the enzyme and is facilitated by an electron transfer system with oxygen and NADPH, similar to that described for $P450_{scc}$ (Hanukoglu, 1992). There are two possible substrates for this sequence: pregnenolone (a Δ^5 steroid) producing dehydroepiandrosterone (DHEA), or progesterone (a Δ^4 steroid) producing androstenedione, the precursor to testosterone. The use of the Δ^5 or Δ^4 substrate is dependent on the C_{17} - C_{20} lyase species e.g. the human enzyme has extremely low C_{17}, C_{20} -lyase activity toward Δ^4 progesterone, compared to Δ^5 pregnenolone so favours the Δ^5 steroidogenic pathway. The opposite is true in the rat (Fluck et al., 2003; Swart et al., 1993).

$P450_{C17}$ expression is induced following stimulated increases in cAMP levels, implying different regulatory mechanisms are in place than for $P450_{scc}$ and 3β-HSD (Payne and Youngblood, 1995). For example, $P450_{C17}$ protein expression was reduced but 3β-HSD levels were unaffected in testes from fetuses exposed in utero to oestrogenic compounds (Majdic et al., 1997b). Additionally, in vitro studies with the protein synthesis inhibitor cyclohexamide demonstrated that $P450_{C17}$ transcription is mediated via cAMP and requires the synthesis of de novo proteins (i.e. cAMP responsive region (CRR) binding proteins) to a greater extent than for $P450_{scc}$ transcription, suggesting different regulatory mechanisms exist for the transcription of the two proteins (Payne and Youngblood, 1995). Activation of AMH–receptor type II (a Sf-1dependent protein) has recently been shown to inhibit postnatal PKA mediated expression of $P450_{C17}$ via a cAMP regulatory element-binding (CREB) protein independent mechanism, leading to inhibition of steroidogenesis (Fynn-Thompson et al., 2003).

Quantification of the $P450_{C17}$ expression in human fetal testis samples (13-26/40weeks of gestation) showed that $P450_{C17}$ mRNA was most abundant in the adrenal, followed by testis and ovary, but was undetectable in the placenta, kidney, liver, spleen, intestine, or muscle. The $P450_{C17}$ mRNA reached its peak between 14-16 /40w and showed diminished levels by 26/40w (Voutilainen and Miller, 1986). In the rodent, $P450_{C17}$ mRNA is detected in the mouse urogenital ridge from $e_m10.5$ and in the differentiated testis from $e_m12.5$ throughout gestation as for $P450_{scc}$ (Payne and Youngblood, 1995).

1.1.6.3.4 17β-HSD

Seventeen beta-hydroxysteroid dehydrogenase (17β-HSD, also known as 17-ketosteroid reductase, 17KSR) is found in the smooth endoplasmic reticulum of the Leydig cell. In the Leydig cell, it catalyses the reduction of androstenedione to testosterone, the final step in testosterone biosynthesis (Payne and Youngblood, 1995). This is a reversible reaction, dependent on the levels of substrate and co-factors, namely androstenedione and NADPH for testosterone synthesis (Hanukoglu, 1992).

There are multiple isoforms of 17β-HSD. Studies in mice have shown changes in the localization of isoform expression and consequent testosterone secretion occur during development. For example, the type 3 isoform (the main reductive isoform in the testis) was expressed only in the seminiferous tubules in fetal testes but in the interstitial tissue in adult testes. Expression of the type 3 isoform declines in the seminiferous tubules before puberty but then reappears in the developing adult Leydig cell population. Production of testosterone up until puberty is dependent upon 17β-HSD activity in the seminiferous tubules: a "two cell" requirement for fetal testosterone synthesis (O'Shaughnessy et al., 2000). It is

increasingly clear that in the mouse, Leydig cells in the fetal/neonatal testis lack significant levels of any isoforms of the enzyme capable of reducing androstenedione to testosterone and appearance of this enzyme in the interstitial tissue is due to development of the adult Leydig cell population. Androstenedione is a weak androgen and data show that formation of the bioactive 17β-hydroxy-$_{C19}$ steroids in the fetal/neonatal testis is dependent on 17βHSD localized in the seminiferous tubules, probably within the Sertoli cells (O'Shaughnessy et al., 2000).

To examine regulation of 17β-HSD type 3 mRNA expression in the testis, mRNA levels were measured during development in normal mice and in mice lacking circulating gonadotrophins (hpg) or functional androgen receptors (Tfm). During neonatal development expression of 17β-HSD type 3 is independent of gonadotrophin action, while the increase in expression at puberty is primarily dependent upon androgen action although testicular descent and gonadotrophins are also required (Baker et al., 1997).

Overall, fetal Leydig cells appear in the interstitium of the developing testis just as the seminiferous cords form, characterised by the appearance of large mitochondria and lipid droplets and copious amounts of smooth ER. Fetal rat testosterone production is evident in vivo from e15.5, though from e13.5 the testes can convert progesterone to testosterone in vitro. The ability of the Leydig cell to bind LH starts after e15.5, though the coupling of cAMP with steroidogenic responsiveness is functional before that (Saez, 1994). The role of the LH and cAMP signalling is detailed in section 1.2.

Mutation of any of the genes for the four steroidogenic enzymes discussed above, leads to compromised testosterone synthesis, such as lipoid CAH as with StAR mutations, and psuedohermaphroditism because of failure of normal masculinisation during development (Hanukoglu, 1992; O'Shaughnessy et al., 2000; Payne, 1997; Stocco, 2002).

As Sertoli cells differentiate prior to the Leydig cells, and probably perform the last step of fetal testosterone biosynthesis in the mouse, it appears that the Sertoli cells really are the orchestrators of testis formation and development.

1.1.7 Embryological events in male sex differentiation

This review has described the development of the testis mostly in the mouse but also in the rat and with occasional reference to the human. At birth, the human and rat testes have already achieved an organised structure with defined cell types and vasculature, ready to grow and differentiate further into a mature hormone- and gamete-producing organ. The

most fundamental difference in the development of this organ between these three species is the timescale over which it occurs. For clarity, the major events of development are tabulated per species below (Table 1.2), above a simplified time-line for development, using the human as an example (Figure 1.6).

Species	Stage of testis development			
	Genital ridge	Bipotential gonad	Sry expression	Initial testis cord formation
Human (week of gestation/ 40w)	5	6	7	7-8
Mouse (embryonic day (e_m))	9-10	10-11.5	10.5	11.5-12.5
Rat (embryonic day (e))	10-11	11.5-12.5	12	13.5-14.0

Table 1.2 Gestational age at each stage of testis development in humans, mice and rats. Taken from "Sertoli cell biology" (Edited by M.K.Skinner and M.D.Griswold, 2005).

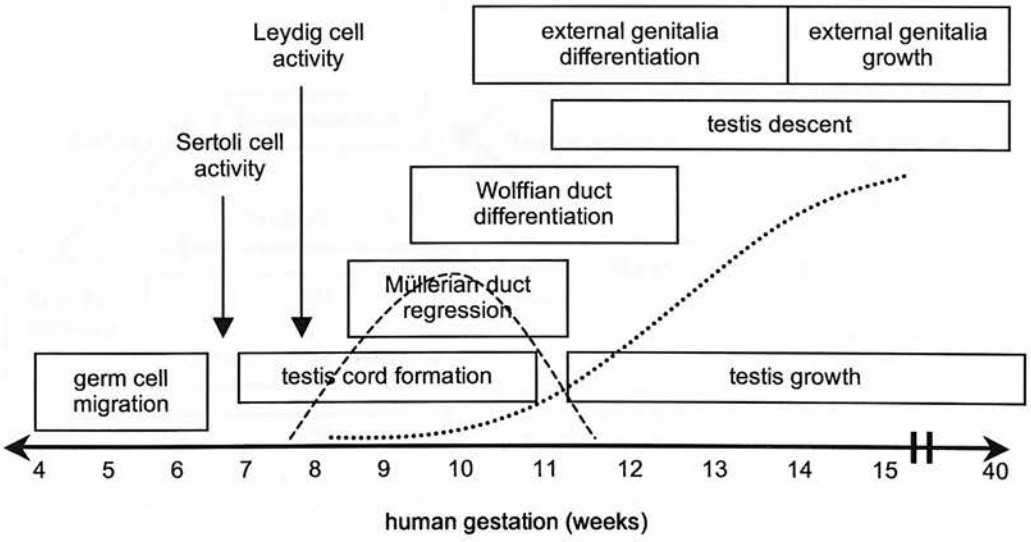

Figure 1.6 Temporal events in testis development in the fetal human over the 40 weeks of gestation. The dashed lines represent: (---) AMH and (...) testosterone production levels, following differentiation of Leydig cells from 8 weeks, which peaks at around 12-16 weeks. Based on (Hughes, 2001) and (Voutilainen, 1992).

1.2 Endocrine regulation of testis development

Testis development requires the correct spatial and temporal expression of many genes. In part, the regulation of this highly complex sequence is under the control of hormones. In

turn, the sexual differentiation of the developing gonad is confirmed by its own hormonal secretions (Capel, 1998). The release of a hormone from one cell, to act on another cell located remotely from the origin, is performed by endocrine cells, naking the testis an endocrine organ.

1.2.1 The HPG axis

Many endocrine organs influence the reproductive tract, including the thryoid and the adrenals, but three tissues are vital to the regulation of the endocrine health of the reproductive system: the hypothalamus, the anterior pituitary and the gonads. Together, they form the Hypothalamic-Pituitary-Gonadal (HPG) axis (Figure1.7).

For the male, the stimulation of production of hormones from the testes is initiated in the hypothalamus of the brain. The hypothalamus synthesises a peptide hormone, gonadotrophin releasing hormone (GnRH), that travels via the portal blood system to the anterior pituitary. GnRH is released in a pulsatile manner, stimulating rhythmic release of the gonadotrophic hormones from the pituitary. The male pituitary releases two hormones into the blood: luteinising hormone (LH) and follicle stimulating hormone (FSH). These are named after their roles in female reproduction but identical forms exist in the male.

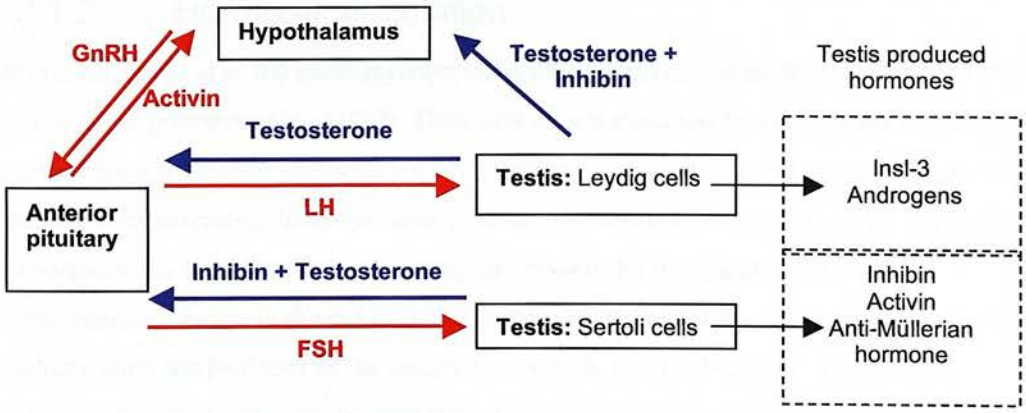

Figure 1.7: Outline of the HPG axis in the male. Certain hormones stimulate (red) production of other hormones; others inhibit (blue) production when their circulating level reaches a critical concentration. The balance of stimulation and inhibition is the regulatory mechanism that underlies this axis.

1.2.1.1 Gonadotrophin release

Regulation of gonadotrophin secretion involves complex interplay between GnRH and feedback regulation by gonadal sex steroids (e.g. testosterone) and peptides (e.g. inhibin-B) as well as pituitary factors (e.g. activin) (Figure 1.7) (Blumenfeld and Ritter, 2001).

GnRH stimulates the secretion of LH and FSH from pituitary gonadotroph cells, therefore it is considered the central regulator of gonadotrophin dependent regulation of the reproductive system. At least two forms of GnRH exist, each capable of regulating different pituitary endocrine cells, enabling the differential stimulation of LH or FSH secretion. The gonadotrophins are released from the anterior pituitary following GnRH stimulation. This triggers secretory granules, formed from the trans-Golgi network and containing the regulatory proteins, to fuse with the cell membrane, dispersing their contents in the extracellular space (Tooze, 2001).

The hpg mouse is a model for hypogonadotrophic hypogonadism due to a deletion in the GnRH gene, preventing formation of a functional GnRH protein. These animals show that pre-natal GnRH expression is vital to enable the necessary development of the neural connections needed for normal hypothalamic-pituitary interactions (Markkula, 1996). Pituitary LH synthesis can start in the absence of GnRH but is dependent on GnRH for a normal level of synthesis and secretion. The undetectable level of LH in the circulation of the hpg mouse despite the development of LH synthesis in the pituitary shows that GnRH is essential for LH release from the pituitary. It should, however, be noted that although circulating LH is undetectable in the hpg mouse, there might be low basal secretion from the pituitary. FSH data was not reported (O'Shaughnessy et al., 1998).

1.2.1.2 Hormone stimulation

LH and FSH belong to the same glycoprotein family as thyroid stimulating hormone (TSH) and chorionic gonadotrophin (hCG). Their actions are executed following their binding to specific receptor proteins on the target cells surface. Many cell surface receptors respond to stimulation by activating heterotrimeric guanine nucleotide binding proteins (G-Proteins). Activation of an expressed G-protein coupled receptor by its ligand results in a conformational change in the cytoplasmic domain of the receptor and the dissociation of the α subunit from the $\beta\gamma$ dimer of the coupled G-protein. Both subunits are then free to modulate intracellular targets. Several ligands modulate multiple effectors as single receptors can interact with multiple G-proteins via differential coupling preferences. Usually it is the α subunit that determines the typical consequence of the receptor binding and the classification of the G-protein e.g. G_s- coupled receptors stimulate adenylyl cyclase via GTP hydrolysis, G_i- coupled receptors inhibit adenylyl cyclase and G_q- coupled receptors stimulate phopholipase C (PLC) (Zhu, 1996).

Co-ordination of the stimulation / inhibition of adenyl cyclase regulates the concentration of small intracellular signalling molecules, such as cyclic adenine mono-phosphate (cAMP). Adenyl cyclase is a membrane bound enzyme responsible for the formation of cAMP from

its precursor molecule adenine tri-phosphate (ATP). The de novo synthesised cAMP is rapidly destroyed by competing enzymes, cAMP-phosphodiesterases, which hydrolyse it to inert 5'AMP, hence its effectiveness as a transient signal. cAMP goes on to activate the enzyme cAMP–dependent protein kinase A (PKA), which catalyses the phosphorylation of selected proteins, regulating their activity. Specific phosphatase enzymes de-phosphorylate the effects of PKA. The substrates of PKA are cell specific, hence the potential for a diverse range of acute responses via the same second messenger molecule (reviewed in Alberts, 1994 and Saez, 1994). The rise in cAMP induces three responses: lipid synthesis, protein synthesis and/or phosphorylation of specific proteins to modify their function (Lacapere and Papadopoulos, 2003).

1.2.1.3 Luteinising Hormone

LH action is exerted on cells expressing the LH receptor. In the testis LH is the main hormone that controls Leydig cell function. LH binding to the Leydig cell activates adenylate cyclase (and phospholipase C to a lesser extent) to increase cAMP production within the cell, that stimulates steroidogenesis and leads to an increase in testosterone production and secretion (Habert et al., 2001). hCG exerts 'superagonist" properties on the LH receptor, inducing a greater level of testosterone production than equivalent amounts of LH (Zhou and Hutson, 1995). Quite how this superantagonism effect works is unclear.

The adult rat Leydig cell can have either an acute or a chronic response to LH stimulation. The short-term reaction sees a sharp increase in the levels of steroidogenesis by the Leydig cell, associated with altered StAR expression, whereas longer-term effects are induced on Leydig cell structure and function. At low doses, LH stimulates the expression of the steroidogenic proteins but long-term high doses induce desensitisation of the steroidogenic response to the trophic hormones, perhaps due to the androgen repressed $P450_{C17}$ expression (Payne and Youngblood, 1995; Sharpe and Cooper, 1987).

The fetal Leydig cell responds to LH with an increase in cAMP and testosterone production, via a G-protein coupled receptor, as early as 15.5 days of gestation in vitro. Although G_s protein is functional in the fetal Leydig cell as early as 14.5 days of gestation, there is no measurable LH receptor in the fetal rat testis at this age, preventing stimulation via this transmembrane signaling system in the fetal Leydig cell (O'Shaughnessy et al., 1998; Warren, 1989). Testosterone produced prior to the establishment of the LH responsive signalling mechanism is therefore gonadotrophin independent, suggesting either that the Leydig cells are constitutively

active during early development or that an unknown factor maintains and stimulates Leydig cell function during at least part of fetal life (O'Shaughnessy et al., 1998). Possible factors

are being investigated, with potential candidates including vasoactive intestinal peptide (VIP) and pituitary adenylate cylclase-activating polypeptide (PACAP-27), both of which stimulate fetal but not adult Leydig cells (El-Gehani et al., 1998a; El-Gehani et al., 1998b). Study of fetal pituitary LH levels and Leydig cell function in hypogonadal (hpg) mice that lack GnRH and, thus, circulating gonadotrophins revealed that LH receptor mRNA was detected in fetal testes from e16 in both normal and hpg mice. In hpg mice, intratesticular testosterone levels were normal throughout fetal development until the day of birth, but were barely detectable by postnatal d5. Together these data confirm that fetal Leydig cell function in the mouse is normal in the absence of LH but they become dependent on LH shortly after birth (O'Shaughnessy et al., 1998). These observations have been complemented by studies with decapitated rat fetuses that also showed fetal Leydig cells produced testosterone without LH stimulation (Migrenne et al., 2001).

In the human, testosterone is detected in the testis by 7w/40w of gestation, following the initiation of sexual differentiation at the start of week six, peaking between 15-18w/40w. However, pituitary gonadotrophins are not detected until 11-12w/40w (Saez, 1994). Placental hCG may regulate testosterone synthesis intermediately, during 15- 22w/40w, in parallel with the surge in testosterone production known to occur during the same period of development (Molsberry et al., 1982). LH and FSH are proposed to have regulatory effects on fetal gonads during the last third of gestation. (Molsberry et al., 1982; Voutilainen, 1992). Mutation of the LH-receptor in humans results in a range of phenotypes from complete sex-reversal to micro-penis (Hughes, 2001).

1.2.1.4 Follicle stimulating hormone

FSH action is exerted on cells expressing the FSH receptor, exclusive in the testis to the Sertoli cell. FSH binding to adult Sertoli cells activates adenylate cyclase to increase cAMP production within the cell. In turn, this results in the phophorylation of the transcriptional activator CREB protein (cAMP response element binding protein). Fetal rat Sertoli cells do not appear to respond to FSH but do express the FSH receptor after e18.5 (Rouiller-Fabre et al., 2003). The presence of FSH receptors in fetal human testes, from 8w/40w has been shown (Plant and Marshall, 2001). In the rat, during perinatal development, FSH concentrations influence the rate at which the immature Sertoli cell proliferates and possibly stimulates release of a paracrine factor from the Sertoli cells to stimulate Leydig cell function (Markkula, 1996; Migrenne et al., 2003). In the adult testis this is balanced via a feedback loop with the Sertoli cell product inhibin-B, which suppresses FSH gene expression, and in turn might be regulated by germ cells, according to their number and differentiation stage (Plant and Marshall, 2001).

1.2.2 Secondary sexual characteristics

Premature activation of the HPG axis presents as central precocious puberty in children, due to a premature stimulation of pubertal gonadotrophin release by GnRH. This results in pubertal serum levels of sex steroids and development of secondary sexual characteristics, such as the growth of external genitalia (Sehested et al., 2000). The loss of normal endocrine communication along the HPG axis can result in an altered correlation between the phenotype and the genotype of an individual, described as hermaphroditism.

Normal development of male external genitalia is dependent on endocrine activity as the presence of androgens converts the bipotential genital primordium into the penis shaft, glans penis and scrotum and facilitates the descent of the testes from their initial high abdominal position through the inguinal canal to the scrotum. Normal development of female genitalia is sex-steroid independent, to the point that even if the ovaries are removed, the external genitalia develop normally (Olaso and Habert, 2000).

1.2.2.1 Testis descent

Relocation of the testis from its origin, by the base of the kidney high in the abdominal cavity, to the scrotum is a two phase process: transabdominal then inguino-scrotal descent (Hutson et al., 1997). Transabdominal migration is influenced by various factors but is primarily mediated by the enlargement of the gubernaculum via a combination of insulin-like-factor-3 (Insl-3) and testosterone/ DHT and the regression of the cranial suspensory ligament (CSL) via an androgen dependent process (Hughes, 2001). The ultimate positioning of testes and ovaries is the result of the sexually dimorphic development of the CSL and the gubernaculum as well as hormone production levels. In the male, the gubernaculum, a mesenchymal tissue, thickens and contracts becoming bulbar and so tethers the testis at the caudal end of the abdominal cavity during elongation of the fetus, from e15.5 to birth (Shono et al., 1994). In the female, development of the CSL and absence of gubernacular growth maintains the ovary beside the kidney.

Gubernacular enlargement is also seen in female mice that over-express Insl-3, where normally it would remain a thin cord in the female (Adham et al., 2002). Insl-3 $^{-/-}$ males present with maldescended testes, cryptorchidism, as a direct consequence of defective gubernacular growth (Boockfor et al., 2001) (Figure 1.8). Insl-3 is produced by the fetal Leydig cell and its receptor (LGR-8) is located in the gubernaculum (Kubota et al., 2002). Following birth and under androgen regulation, the mesenchymal cells of the gubernaculum disappear while the muscular layer invaginates towards the developed scrotum, creating the processus vaginalis, also called the cremasteric sac. Contraction of the inverted cremaster

muscle and the intra-abdominal forces the testes into their mature location: the scrotum. Unlike in the human, the rat processus vaginalis does not narrow, enabling descended testes to be retracted, if required. Complications of undescended testes include infertility and increased risk of testicular malignancy (Adham et al., 2002).

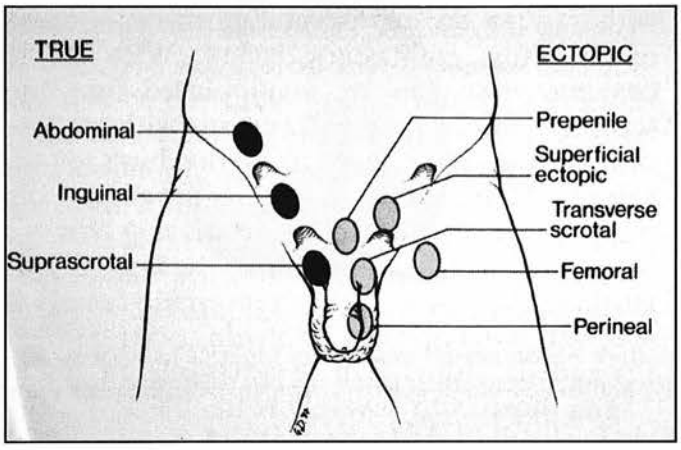

Figure 1.8 Illustration of testis descent, from normal fetal positioning in the abdominal region to adult scrotal position. Examples of ectopic testis positions, cryptorchidisms are also shown. Image found on-line via www.google.com.

1.2.2.2 Penis formation

In mammals, fertilisation requires specially formed external genitalia. These develop from the genital tubercle, a ventrocaudal structure composed of bilateral shelves of mesenchyme bisected by a central epitheliuml plate. The transformation of this primordium into a functioning penis is mostly androgen dependent (Morgan et al., 2003). Disturbances in the signalling associated with normal penis development can result in the incomplete fusion of the urethral folds during perinatal development, resulting in the malpositioning of the urethral opening along the penile shaft or on the scrotum, hypospadias (Bianca et al., 2003). Hypospadias can affect any of several different regions of the developing glans penis, ranging from slight changes (coronal hypospadias) to proximal defects (perineal hypospadias) (Figure 1.9). The mechanisms behind normal and maldevelopment of the genital tubercle are still being investigated. These include androgen dependence as well as other, less defined, factors as highlighted by recent studies with Hoxa13 mutant mice (Morgan et al., 2003).

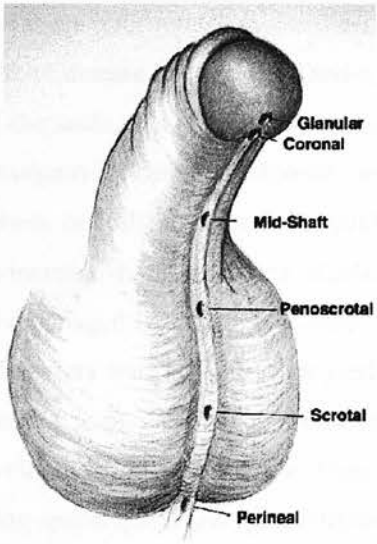

Figure 1.9 Illustration of urethral malclosure on the penile shaft, hypospadias. Note mid-shaft hypospadias also described as penile. Image found on-line via www.google.com.

1.2.2.3 Male accessory glands

The male reproductive tract includes the testes and the penis but also the epididymis, the prostate and the seminal vesicles. The epididymis extends from the efferent ductules to the vas deferens and plays a vital role in regulating the development of sperm motility and fertilising capacity as well as being a storage area, in some species, for mature spermatozoa pending ejaculation (Rodriguez, 2001). Along with the other Wolffian structures, the seminal vesicles and vas deferens, the epididymis derives from embryonic mesoderm. The prostate envelops the urethra by the bladder and has a secretory role. It is derived from the embryonic endoderm. Androgens are required for prostatic growth and development as well as to initiate and maintain its secretory activity in adulthood (Thomson, 2001).

1.3 Testicular dysgenesis syndrome

Fetal development has a crucial influence on postnatal development and life-long related disease risk. For example, perturbed fetal growth is associated with increased risk of coronary heart disease and type II diabetes (Davies and Norman, 2002). Increasing reports in the literature extend this observation to indicate that external influences i.e. environmental exposures, can also affect fetal growth (e.g. cigarette smoking) as well as mis-programme specific stages of development, resulting in permanent changes in structure, physiology and metabolism of the target organ. The physiological mechanisms through which environmental influences are transmitted to the target organs are many and diverse. Any external influence

that insults the establishment of these inter-related systems can result in mal-development, contributing to an increased risk of disease in later life (Davies and Norman, 2002).
Regarding the development of the testis, this is a complex process associated with hormonal regulation via many endocrine organs including the thyroid, pituitary, hypothalamus, the adrenals and the testes themselves. In 2001, an article was published that applied a comprehensive analysis of the increase in the incidence of disorders of male reproductive tract development, traditionally managed across several different medical disciplines. It hypothesised that though the disorders were typically analysed separately following clinical presentation, they shared a common theme: they were all the result of disrupted programming and gonadal development during fetal life. Together, the four disorders (testicular cancer, low/ declining sperm quality, cryptorchidism and hypospadias) were symptoms of a testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001).
Some of the evidence contributing to Skakkebaek's hypothesis, of the shared fetal origin for the four disorders of the human male reproductive system, is reviewed here.

1.3.1 Testicular cancer

In 1994, Adami et al reported their analysis of the incidence of testicular cancer among 10 European cancer registries. During the entire period of registration (maximally between 1943-1989), incidence increased rapidly in all countries, by 2.3-3.4 % annually in the Nordic countries and by about 5% in Poland and Germany. The rising trend was most pronounced in men below 30 years old. The age-specific incidence peaked in all countries at ages 25 to 34, but the geographical variation was considerable (Adami, 1994). These data indicated that environmental influences on testicular cancer are strong and demanded new aetiological hypotheses, such as set out in Skakkebaek's 2001 paper. Danish data on testicular cancer collected between 1943-1996, along with prostate cancer data, showed an annual increase in testis cancer of 2.6% compared to 1.6% for prostate cancer. This data also revealed that the epidemiological pattern seen with prostate cancer could be explained by changes in diagnosis and disease registration but this could not account for the change in testis cancer incidence which was associated with causal factors (Moller, 2001).
Testicular germ cell tumours are the most common cancer in men aged between 20-45years. They arise from precursor premalignant cells, carcinoma-in-situ (CIS) cells, which are believed to derive from primordial germ cells that failed to undergo normal differentiation during fetal testis development (Rajpert-De Meyts et al., 1998). Ultrastructural studies suggest that the pathogenesis of germ cell neoplasia involves excessive proliferation of precursor germ cells associated with loss of intercellular communication (Gondos, 1993). It is hypothesised that if the development of the testis is disturbed or delayed, primordial germ

cells or gonocytes undergo maturation delay or differentiation arrest, which may render them susceptible to neoplastic transformation. Furthermore, the phenotypical heterogeneity of CIS cells may be associated with their potential to give rise to different tumour types and may be related to the developmental stage of the early germ cell which has undergone malignant transformation (Rajpert-De Meyts et al., 1998).

There are no reliable imaging techniques or serological parameters that can diagnose CIS leaving the only means of accurate diagnosis as surgical biopsy of the testis. A biopsy sample is fixed and subjected to immunhistochemical staining for placental alkaline phosphatase (PlAP), not usually detectable in normal adult germ cells (Dieckmann and Skakkebaek, 1999). Early detection of CIS enables their eradication by low-dose irradiation but established germ cell tumours can only be cured by removal (i.e. orchidectomy) or irraditation (Mosselman et al., 1996).

CIS cells are associated with other clinical conditions, including male infertility, cryptorchidism and low sperm counts. Background levels of CIS are less than 1% among infertile men but increase to 4% when associated with cryptorchidism. Where ambiguous genitalia are presented, up to 25% of patients also have CIS in their testes, consistent with the high risk of testicular cancer associated with this clinical condition (Dieckmann and Skakkebaek, 1999). A recent study prompted by the Skakkebaek hypothesis investigated whether testicular germ cell cancer may be aetiologically linked to other male reproductive abnormalities as a part of the TDS. CIS was detected in 8.7% of biopsies from contralateral testes in patients with a testicular germ cell tumour. The cumulative incidence of one or more signs of microscopic testicular dysgenesis was 25.2%, supporting the hypothesis that this cancer is part of a testicular dysgenesis syndrome (Hoei-Hansen et al., 2003). Increased risk of CIS being present was seen in a separate study following ultrasonic detection of micro-calcifications in the contralateral testis (Holm et al., 2003).

1.3.2 Semen quality

Most commonly and most mildly, TDS presents as impaired spermatogenesis and only rarely with the full range of disorders in a single patient (Skakkebaek et al., 2003). Reports of a decrease in semen quality of men in four European countries has suggested regional differences. A study was undertaken of semen samples from fertile men from four European cities (Copenhagen, Denmark; Paris, France; Edinburgh, Scotland; and Turku, Finland). Finnish men had the highest sperm counts but men from Edinburgh had the highest proportion of motile spermatozoa (Jorgensen et al., 2001). The Danish population was generally the worst affected and further reports state that >30% of young Danish men have sperm counts in the sub-fertile range, a change that is not accounted for entirely by social

factors (Jensen et al., 2002). In Denmark, 5% of children are born after assisted reproduction (Skakkebaek, 2002). Even if a secular trend cannot be demonstrated to be biologically significant, the data regarding regional differences are more convincing (Jensen et al., 2002). These differences in semen quality may indicate different environmental exposures or lifestyle changes in the various populations (Jorgensen et al., 2001).

1.3.3 Cryptorchidism

The maldescent of the testes, cryptorchidism, is the most common congenital anomaly in humans, affecting up to 5% of male infants (Adham et al., 2002). In Western countries, 1-2% of males at the age of 3 months are diagnosed with this condition but its aetiology is still unclear. Potential causes might include physical obstruction of the descent, endocrine abnormalities or genetic mutations associated with the gubernaculum (Raivio et al., 2003; Roh et al., 2003). Cryptorchidism can resolve spontaneously in boys up to around 3 months old and of those left, up to 40% recover following hormonal stimulation by hCG (Giannopoulos et al., 2001).

According to two comparable English studies, the incidence of cryptorchidism in full-term boys approximately doubled between the 1950s and the 1980s (~2.7 and ~3.84% at birth and ~0.9 and ~1.4% at 3 months, respectively). Regionally there are large differences: e.g. in Finland the incidence of cryptorchidism (<1%) is clearly lower than in Denmark (~2% at 3 months). Regional and temporal trends may help to identify environmental factors that might be associated with these disorders (Toppari et al., 2001).

Recently, a prospective study of cryptorchidism was reported, that compared the contemporary prevalence of cryptorchidism in Denmark (1997-2001) and Finland (1997-99). 9% of Danish and 2.4% of Finnish boys were found to exhibit cryptorchidism at birth, falling to 1.9 and 1% respectively, by 3 months of age. The conclusion of the paper suggested that while genetic variation might account for this geographical difference, it was considered more likely to be a consequence of different environmental factors exposure (e.g. endocrine disruptors) and lifestyle between the two countries (Boisen et al., 2004). Additionally, these data showed an increase in cryptorchidism in Denmark over the past 40 years (1.8% in the 1950's), consistent with Skakkebaek's hypothesis (Boisen et al., 2004).

1.3.4 Hypospadias

The malpositioning of the urethral opening, hypospadias, is another common congenital anomaly in humans, affecting up to 0.4% of male infants (Vrijheid et al., 2003). Increasing trends in the incidence of hypospadias were found in Sweden during the 1960s, and in Norway, Denmark, England and Hungary during the 1970s. In Norway and Denmark, the

increase continued in the 1980s, while in the USA it has continued from the 1970s to the 1990s. Finland has shown a lower reported rate of hypospadias than other Nordic countries. However, it is difficult to make comparisons between countries because of variable inclusion criteria, but as with the related reports of cryptorchidism, regional and temporal trends may help to identify environmental factors associated with these disorders (Toppari et al., 2001). A study of the contemporary prevalence of hypospadias in Danish newborn boys (1997-2004) was recently reported (Boisen et al., 2005). The boys were examined 4 times: shortly after birth then at 3, 18 and 36 months and data compared to a concomitant Finnish study. At birth, 1.03% of Danish and 0.27% of Finnish boys were found to exhibit hypospadias. This increased to 4.64 and 1% respectively by 36 months of age. This increase was due to the detection of milder cases that only became evident as normal development progressed and the prepuce could be retracted. The conclusion of the paper suggested that while the aetiology of most hypospadias remains obscure, genetic variation might account for some of the cases seen but it was hypothesised that other cases may be due to intra-uterine exposure to unspecified environmental factors. This approach could partly explain this geographical difference, consistent with Skakkebaek's 2001 hypothesis (Boisen et al., 2005).
Given that historical data for cryptorchidism and hypospadias from international registries may not be totally reliable due to different calling levels, and that sperm count data is still emerging, only the data regarding testicular cancer shows a definite progression towards a significant increase in the symptoms of TDS (Sharpe, 2001). Perhaps with these data in mind, Skakkebaek added to his hypothesis that the increased risk of TDS in recent times may be related to adverse environmental influences during fetal development, accentuating any genetic predisposition of the male reproductive tract to disease. This posed a question: what effects may be expected in humans if those endocrine disrupters, ubiquitous in small amounts in food and water, have an impact on the male reproductive system (Skakkebaek et al., 2001)?

1.4 Endocrine disruption

Experimental and epidemiological studies suggest that TDS is the result of disruption of he hormonal contribution to embryonal programming and gonadal development during fetal life (Skakkebaek, 2003). Endocrine disrupters (ED's) are environmental substances that mimic/ antagonize/ or alter normal endocrine signalling. Changes in endocrine programming inflicted during early development, can permanently affect the physiology of the fetus, perhaps predisposing it to adult onset disease (Davies and Norman, 2002).

The physiological mechanisms through which environmental influences are transmitted to the target organs are varied and complex. Environmental agents can act as weak oestrogens or anti-androgens and are believed to contribute to the increase in impaired sex determination, reduced sperm counts and reduced fertility reported in many wildlife species (Skakkebaek et al., 2001). Though the testis itself undergoes fetal development without hormonal stimulation, the hormones it produces (e.g. testosterone, AMH, Insl-3) are vital for normal development of all other aspects of masculinisation. Any chemical with the ability to interfere with the production of these factors, and others, has the potential to mis-programme the masculinisation of the fetus (Sharpe, 2001).

1.4.1 Examples in wildlife

Accidental exposure of wildlife to unusually high levels of endocrine disrupters following environmental disasters has highlighted the effect these chemicals can have. For example, a study in the early 1990s reported a number of abnormalities in the hatchling and juvenile alligators of Lake Apopka, Florida, USA. This lake had been contaminated following a spill of DDT which added to existing contamination by local agricultural activities and nearby sewerage treatment facilities. Lake Apopka juveniles had significantly elevated serum concentrations of many synthetic compounds including p,p'-DDE, DDT and PCBs compared to juveniles from the other lakes (Guillette et al., 1999). Juvenile male alligators living in/ around the contaminated lake were found to have reduced levels of plasma testosterone compared to control males from a nearby but uncontaminated lake (Lake Woodruff), as well as poorly organized testes and abnormally small phalli (Guillette et al., 1994). These observations were followed up with in vitro experiments examining the steroidogenic activity of gonads removed from juvenile alligators obtained from contaminated or control lakes in central Florida. Testes from males obtained from the contaminated lake synthesized significantly higher concentrations of oestradiol when compared to testes obtained from control males (Guillette et al., 1995). The effects seen in these studies were hypothesised to be due to chemical exposures during embryonic development rather than a consequence of levels of contaminants in the juvenile serum (Guillette et al., 1999).

Studies with captive reptiles, whiptail lizards (Cnemidophorus inornatus) investigated the source of individual variation in intensity of sexual behaviours in captive males. The data suggested that individual variation in the sexual behaviours exhibited was not due to low circulating concentrations of androgens or different profiles of testicular steroidogenesis but considered either an inability to respond to androgens or an inability to exhibit sexual behaviour (Lindzey and Crews, 1992). These lizards were not bred in captivity, implying that any purtubations in their sexual behaviour must have occurred prior to capture. This

may reflect the level of ED contamination present in the environment from where they originated, as even following castration and androgen replacement, low intensity courters continued to exhibit weak and infrequent sexual behaviour. Chemicals that affect breeding performance alone (polychlorinated biphenyls (PCBs), fenarimol, or losulazine), without altering sperm and testicular measures have been reported (Gray et al., 1989).

Portelli et al (1999) tested whether the pesticide 1,1,1-trichloro-2, 2-bis(p-chlorophenyl) ethane (DDT) and its metabolite dichlorodiphenyltrichloroethane (p,p'-DDE), as well as oestradiol-17β, were able to induce feminization of the common snapping turtle (Chelydra s. serpentina). Normally this species of turtle relies on incubation temperature to regulate the sexual differentiation of its eggs. When eggs were incubated at a male-producing temperature (26°C), oestradiol induced female development at this temperature, but p,p'-DDE did not affect sex determination, even at exposure levels comparable to those found in moderately contaminated sites in the Great Lakes (Portelli et al., 1999).

Sewage is considered a major source of environmental pollution and contains a complex mixture of organic and inorganic chemicals (Jobling et al., 1995). Discharge effluents from sewage treatment plants have been highlighted as responsible for the feminization of fish as many EDs occur in effluents, including natural and synthetic oestrogen hormones, degradation products of alkylphenol ethoxylate surfactants, and plasticisers. For example, surface and sediment water samples were collected from the Tyne and Tees estuaries (UK) and analysed for their oestrogenic content. The Tyne and Tees estuaries represent estuaries that have been historically contaminated by industrial activities, and continue to receive treated domestic sewage and industrial effluent. In 1998, Dabholm Gut on the Tees received a mixture of treated and untreated effluent, while Howdon sewage treatment works on the Tyne discharged primary treated effluents. An oestrogenically active water sample collected from Howdon was shown to contain 17β-oestradiol, androsterone, and unknown oestrogenic compound(s). Most of the activity contained in a sample collected from the Dabholm Gut was due to 17β-oestradiol with additional activity from nonylphenol and (tentatively) di(2-ethylhexyl)phthalate (DEHP) (Thomas et al., 2001). Metcalfe (2001) reported that oestrogenic hormones induced intersex (i.e. testis-ova) when fish were exposed to ng/l concentrations of oestrogenic test compounds for 100 days from hatching. Bisphenol A induced testis-ova at a concentration of 10 micrograms/L, but diethylhexyl phthalate (DEHP) did not induce a response. Results were consistent with oestrogenic responses in the yeast oestrogen screening assay (Metcalfe et al., 2001). Jobling et al (1995) randomly screened 20 chemicals present in liquid effluents. Half the compounds tested appeared able to interact

with the oestradiol receptor, demonstrated by their ability to inhibit binding of 17β-oestradiol to the fish oestrogen receptor (Jobling et al., 1995).

These studies showed that aquatic species are susceptible to adverse effects of endocrine disrupting chemicals. However, as this is not directly applicable to the human situation as we are not aquatic (postnatally), further studies have been carried out using mammalian species under laboratory conditions.

1.4.2 Examples in laboratory experiments

Androgens are important for the structural and functional integrity of the testis and the prostate and this may in part be mediated by the aromatisation of testosterone to the oestrogen, oestradiol (Turner et al., 2001). It has been proposed that reduced androgen action sensitizes the reproductive tract to oestrogens, demonstrating that the balance in action between androgens and oestrogens, rather than their absolute levels, may be of fundamental importance in determining normal or abnormal development of some regions of the male reproductive tract (Rivas et al., 2002). The effects of neonatal exposure to the potent reference oestrogen diethylstilboestrol (DES) or the environmental oestrogen bisphenol A (BPA) on reproductive tract development in male rats were compared against untreated rats during the neonatal period before onset of puberty. Adverse changes to the testis and reproductive tract were induced by exposure to DES, but not BPA. The changes mainly resulted from a combination of high oestrogen and low androgen action as either situation on its own was unable to induce the same changes (Williams et al., 2001). Reproductive tract abnormalities induced in the neonatal male rat by a high dose of DES are associated with reduced androgen receptor expression and Leydig cell volume compared to treatments that interfere with androgen production (GnRHa) or action (flutamide) alone which fail to induce reproductive tract abnormalities or alter AR expression akin to that induced by DES (McKinnell et al., 2001).

The above data demonstrate that neonatal development is highly dependent on hormonal regulation, but though fetal testis development is considered to be largely independent of gonadotrophin regulation, studies looking at fetal exposure to anti-androgens or oestrogens have reported treatment related changes (Rouiller-Fabre et al., 2003). Acute exposure to high levels of DES or 4-octylphenol (OP) or vehicle (oil, control) to pregnant rats on e11.5 and e15.5 produced no difference between fetuses from control and treated mothers at e17.5, except for a reduction in the amount of immunostaining for $P450_{C17}$ and SF-1 detected in Leydig cells from both treatment groups. Oestrogen receptors (ERα) were present in the fetal Leydig cells of all animals (Saunders et al., 1997). The follow-up studies again showed that in both DES- and OP-exposed fetuses, immunoexpression of SF-1 was reduced in

Sertoli and interstitial cells when compared with controls but not in fetal ovaries. These results suggested that Sertoli cell-derived oestradiol may be important in the physiological regulation of SF-1 in the fetal testis, providing a possible mechanism by which inappropriate exposure to oestrogens might alter the genetic cascade that ensures normal development of the testis is via altered expression of SF-1 (Majdic et al., 1997b).

Another study investigated the effect of oestradiol (E_2) and diethylstilbestrol (DES) on the testis from 14.5-day-old rat fetuses in culture. Exposure to DES and E_2 decreased gonocyte number, due to a decrease in the gonocyte mitotic index and a dramatic increase in apoptosis. The effects of DES were more drastic than E_2 but in the presence of the anti-estrogen ICI 182.780 (ICI), the effect of DES was abolished. Sertoli cell number decreased due to a decrease in Sertoli cell proliferation, which was not antagonized by ICI. DES had no effect on FSH-stimulated cAMP production after adjustment for Sertoli cell number. Both oestrogens reduced the levels of basal and LH-stimulated testosterone production in vitro, resulting in the disruption of the balance between androgens and oestrogens (Lassurguere et al., 2003).

The anti-androgenic properties of chemicals with endocrine disruptor potential has become the subject of wider research (Skakkebaek et al., 2001). Anti-androgenic chemicals can be clustered into separate groups, based on the resulting profiles of reproductive effects, due to their altering of masculinisation via different mechanisms (Gray et al., 1999). The profile of changes induced by some potentially antiandrogenic compounds on the rodent reproductive system in vivo was described by Gray et al (1999). These were a mixture of environmental agents and research/ therapeutic agents and included: linuron, a urea-based herbicide that induces prolonged LH hyperstimualtion of Leydig cells resulting in adenoma formation; p,p'-DDE, a metabolite of the pesticide DDT, that binds to the AR in vitro and may be associated with the high level of cryptorchidism seen in the Florida panther via dietary exposure (see Facemire et al 1995, 1997) ; procymidone, iprodione and chlozolinate (dicarboxymide fungicides like vinclozolin); ethane dimethane sulphonate (EDS) and ketoconazole (inhibitors of testosterone synthesis: EDS kills Leydig cells, ketoconazole inhibits cytochrome P450 enzymes); di-n-butyl phthalate (DBP) and diethylhexyl phthalate (DEHP) (phthalates induce malformations in androgen-dependent tissues following in utero exposure but not via an AR associated mechanism) and PCB congener number 169, an aryl hydrocarbon (Ah) receptor agonist like the known endocrine disruptor 2,3,7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (Gray et al., 1999).

In utero treatment with the AR antagonist, flutamide, produces ventral prostate agenesis and cryptorchidism, while finasteride, an inhibitor of DHT (a potent androgen and testosterone

derivative) synthesis, rarely, if ever, induces such malformations. Di-n-butyl phthalate (DBP) alters reproductive development by a different mechanism of action than the AR antagonists, evidenced by the display of an unusually high incidence of testicular and epididymal alterations in the male offspring of exposed dams - effects rarely seen after in utero flutamide treatment (Gray et al., 1999). Exposure to low concentrations of DBP during a short, but critical window of development (i.e., sexual differentiation), impaired development of the male reproductive tract and induced a variety of testicular lesions in Xenopus laevis frogs. Most notably, these lesions persisted into adulthood, long after cessation of treatment with DBP (Dawson et al., 1989).

It should be noted that these effects are only usually observed after administration under laboratory conditions and in high doses. This should mean that similar effects are unlikely to be found in humans except perhaps within small at risk populations with unusually high levels of exposure (Sharpe, 2001).The impact of environmental chemicals on reproductive health has gained much attention over the last decade or so, both from scientists and the media. (Norgil Damgaard et al., 2002). Concern about possible adverse effects caused by the inadvertent exposure of humans and wildlife to EDs has led to the development of screening programmes for endocrine effects. New laws in the USA mean that the Environment Protection Agency (EPA) is required to test pesticides etc for endocrine effects (Gray et al., 1999). The laboratory methods needed to identify these compounds are being developed (O'Connor et al., 2002).

1.4.3 Effects on man

Disturbances of hormonal regulation during fetal or postnatal development in humans may induce adverse effects on the male reproductive system, but these adverse effects of endocrine disruptors on humans are subtle, and difficult to research and detect. Based on current knowledge, the impact of endocrine disruptors on male reproductive function remains to be quantified. Monitoring of environmental chemicals in Japan, for example, has already revealed that several endocrine active chemicals are in river water, sediments, and wildlife as well as in the human umbilical cord (Iguchi et al., 2002). While animal studies provide us with valuable insights into the range of effects that can be attributed to EDs following in utero exposure, the concentration of EDs representative of fetal exposure levels is uncertain. Confounding factors include: the ability of chemicals to bioaccumulate in body lipid, the metabolism of body lipid during pregnancy/lactation releasing the mothers lifetime legacy of potential EDs into circulation and the poorly understood kinetics of ED transfer across the placenta (Murray et al., 2001). Assessment of prenatal polychlorinated biphenyl (PCB) exposure, by measuring levels in umbilical cord of women who consumed fish caught

in the Great Lakes, USA, revealed that consumption of fish from Lake Ontario increased the risk of prenatal exposure to the most heavily chlorinated PCB homologues. PCBs are recognised as an environmental pollutant though they were banned in the 1970s from industrial use, but are highly resistant to environmental degradation. They bioaccumulate in fat tissue, especially in marine life including fish at the top of the food chain, and cross the placenta providing in utero exposure as well as becoming concentrated in breast milk fats providing neonatal exposure (Stewart, 1999).

Puerto Rico has the highest known incidence of premature breast development (thelarche) ever reported in girls younger than 8 years of age without other manifestations of puberty. Organic pollutants, including pesticides and some plasticizers, have been widely used in Puerto Rico. Significantly high levels of various diester phthalates and the metabolite MEHP were identified in 68% of the samples from thelarche patients, compared to only one control sample. This study suggests a possible association between phthalates and premature breast development in a human female population, though no mechanism is yet known *via* which phthalates could affect breast development in this way (Colon et al., 2000).

Extrapolation of effects from rodents to humans will be enhanced if future studies incorporate determination of tissue concentrations of the active metabolites. Knowledge of the tissue concentrations of the active toxicants also would provide an important link to in vitro studies, which can provide more useful mechanistic information when they are executed at relevant concentrations (Gray et al., 2001). The relevance of animal study findings to observations in man await a greater understanding of the physiological roles of androgens and oestrogens in normal males and a thorough evaluation of the sources, routes of exposure, concentrations in vivo and bioavailability of chemicals that are potential endocrine disruptors (Saunders et al., 1997).

Epidemiological human studies are necessary to fill in these gaps in our knowledge of the mechanisms that underlie ED action in fetal development, in order to elucidate how they exert their effects (Mori, 2001). But until that data is available, the next best thing is to use alternative models to investigate the mechanisms that underlie the chemicals associated with ED changes known so far and explore the effects of exposure to combinations of such compounds (Sharpe, 2001).

1.5 Phthalates

Phthalates are used as plasticisers in PVC products and as solvents in some personal care products, and are among the most common and widely used industrial chemicals (Foster et al., 2001). Their ubiquity in the environment is well known (Jobling et al., 1995). Much of

this review of phthalates will be biased towards Di-*n*-butyl phthalate because it is the focus of the studies presented in this thesis.

1.5.1 Biochemistry

1.5.1.1 Structure and properties

A phthalate is a synthetic chemical, commercially produced by the condensation of alcohols with phthalic anhydride (1,2-benzenedicarboxylic acid), resulting in paired ester groups on a cyclohexatriene ring (Figure 1.10). Hydrolysis of the phthalate reproduces the phthalic acid. The products of the condensation produce at least three isoforms of the phthalate esters (ortho, meta and para) but the generic term of phthalate ester refers to the ortho configuration. Adipate esters are related chemicals, also used as plasticisers, but their structure a four carbon (C_4) chain separates the ester groups rather that the carbon ring. Depending on the "parent" alcohol, respective alkyl groups are formed on the arms of the phthalic ester (Kluwe, 1982). Those with shorter chains are more water soluble, though the class is generally insoluble in aqueous media (Table 1.4).

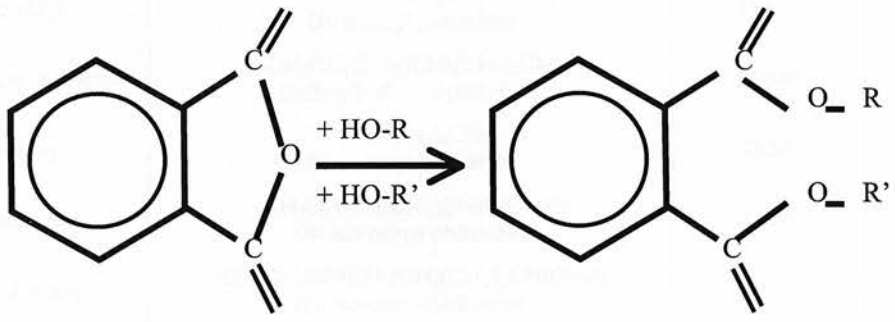

Figure 1.10: A schematic of the condensation reaction between phthalic anhydride and alcohol (+HO-R/R') that creates the common chemical backbone of phthalate structure. The 'R' groups are the alkyl groups from the "parent" alcohol, making the respective di-ester. DBP is formed from n-butanol with phthalic anhydride (Kavlock et al., 2002).

Di-n-butyl phthalate (DBP) has the molecular formula $C_{16}H_{22}O_4$, giving a molecular weight of 278.34. At room temperature DBP appears as pale yellow oil, with a boiling temperature of 340°C and a melting point of -35°C. Its specific gravity is 1.0465, similar to water. It is partially soluble in water but easily soluble in organic solvents. It is also known by its CAS number 84-74-2 and many synonyms: 1,2-Benzenedicarboxylic acid dibutyl ester; benzene-o-dicarboxylic acid di-n-butyl ester; o-benzenedicarboxylic acid dibutyl ester; butyl phthalate; n-butyl phthalate; DBP; dibutyl 1,2-benzene dicarboxylate; dibutylphthalate; di-n-butylphthalate; di(n-butyl) phthalate; dibutyl-o-phthalate; phthalic acid dibutyl ester. Trade Names: Celluflex DBP; Elaol; Ergoplast FDB; Ersoplast FDA; Genoplast B; Hexaplas M/B;

Palatinol C; Polycizer DBP; PX 104; RC Plasticizer DBP; Staflex DBP; Uniflex DBP; Unimoll DB; Witcizer 300; Witicizer 300 (Marsman, 1995).

It has been investigated whether dialkyl phthalates can imitate steroid structure in vitro. Data suggested that the phthalate benzene moiety recognizes the core of the estrogen receptor (ER) binding site, and the hydrophobic interaction of the dialkyl moiety substantiates the binding characteristics of the phthalates. The data indicated that chemicals with slight structural analogy and weak receptor affinity could perturb the endocrine system when administered in high concentrations (Asai et al., 2000).

R / R' structure	Chemical formula of R/R' groups and associated di-ester phthalate name	Di-ester abbreviation	Mono-ester abbreviation
- ethyl	$-CH_2CH_3$ Di-ethyl phthalate	DEP	MEP
- butyl	$-CH_2(CH_2)_2CH_3$ Di- butyl phthalate	DBP	MBP
- cyclohexyl	$-C_6H_{11}$ Di- cyclohexyl phthalate	DCHP	MCHP
- benzyl	$-CH_2C_6H_5$ Di- benzyl phthalate	DBzP	MBzP
- 2-ethylhexyl	$-CH_2CH(C_2H_5)CH_2(CH_2)_2CH_3$ Di-2- ethylhexyl phthalate	DEHP	MEHP
- n-octyl	$-CH_2(CH_2)_6CH_3$ Di- n-octyl phthalate	DOP	MOP
- iso-nonyl	$-CH_2CH_2CH(CH_3)CH_2C(CH_3)_3$ Di- iso-nonyl phthalate	DNP	MNP
- iso-decyl	$-CH_2CH_2CH(CH_3)CH_2(CH_2)_2CH(CH_3)_3$ Di- iso-decyl phthalate	DDP	MDP

Table 1.4 A summary of the most common chemical formulae of the R and R' arms and the names of the resulting phthalates. Adapted from Blount et al, 2000.

1.5.1.2 Absorption and distribution

Their lipophilic nature makes phthalates easily absorbed thorough the skin, though the most common route of adult human exposure is thought to be dietary ingestion. They are generally well absorbed from the gastrointestinal (GI) tract following oral administration. Sub-populations are also prone to high levels of exposure via direct intravenous contact through medical equipment such as DEHP associated with PVC in medical equipment such as blood bags, cannulae or syringes (Blount et al., 2000a). The distributions of DEHP and its metabolite among the protein constituents of human plasma resulting from the leaching of phthalates from blood bags were studied. More than 80 % of the diester was associated with lipoproteins, the remaining diester adsorbed weakly to other proteins, including albumin.

MEHP was in equilibrium between free in solution and adsorbed to albumin; none was bound to lipoproteins (Albro and Corbett, 1978). Because phthalate esters associate with plasma proteins, they are widely distributed in the body. Fat absorptive organs (e.g. GI tract) and excretory organs (e.g. kidney) are considered the main repositories for phthalate di-esters, whereas the monoesters are not accumulated (Kluwe, 1982).

1.5.1.3 Metabolism and excretion

Phthalates can undergo hydrolysis of either or both of their ester groups. Hydrolysis of just one group occurs more readily in vivo, producing a monoester phthalate (Figure 1.11). This is catalysed by esterase enzymes present in many tissues including intestinal mucosal cells. Short-chain esters can be metabolised by extracellular enzymes found in the intestinal contents, e.g. pancreatic enzymes. For the longer-chain molecules such as DEHP, hydrolysis to the corresponding monoester metabolite largely occurs prior to intestinal absorption, then it undergoes oxidative metabolism prior to excretion. Short-chain dialkyl phthalates, such as DBP, can be excreted in an unchanged form or following complete hydrolysis to phthalic acid. It is generally well accepted that orally administered diester phthalates are hydrolysed by lipases in the wall of the small intestine and absorbed almost entirely as the corresponding monoester (Kavlock et al., 2002).

Metabolites of DBP include monobutyl phthalate (MBP), monobutyl phthalate glucuronide, o-phthalic acid and oxidised monobutyl phthalate glucuronide metabolites. MBP-glucuronide is the primary metabolite identified in urine, with ~45% excreted into the bile and ~5% in the faeces (implying efficient enterohepatic recirculation occurs) (Kavlock et al., 2002). Glucuronidation (by β-glucuronidase) has been hypothesised to reduce mono-ester toxicity (Foster et al., 1983; Saillenfait et al., 2001).

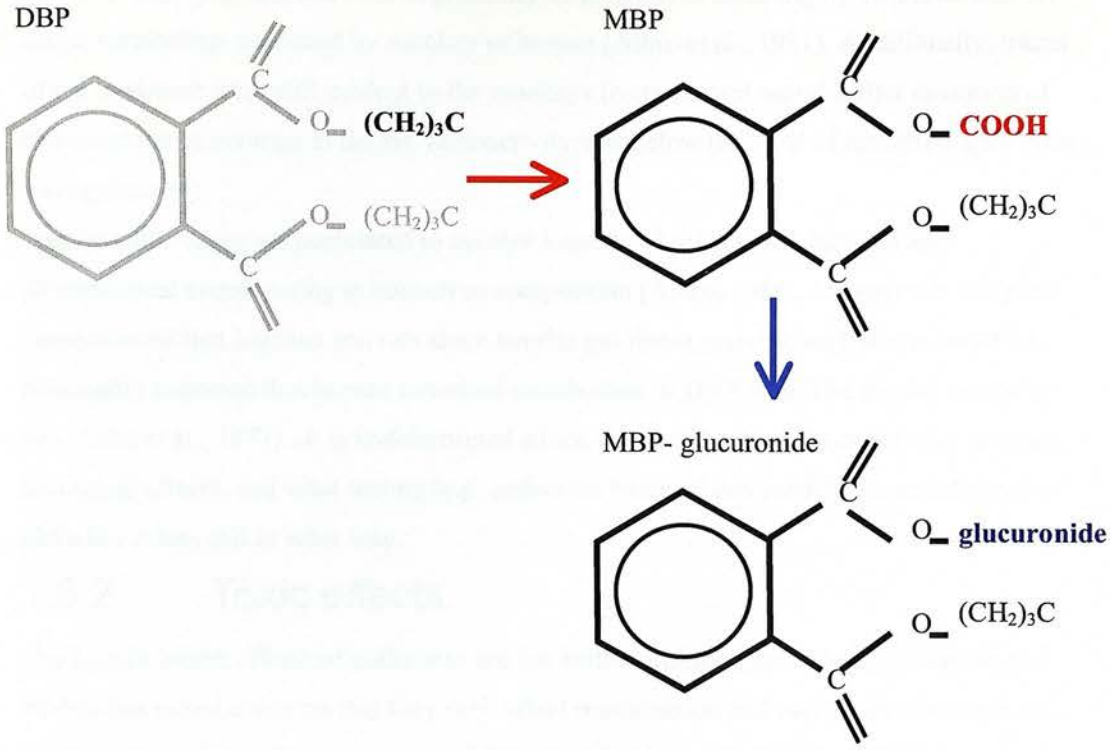

Figure 1.11: Schematic representation of the chemical structure of DBP and the metabolite MBP after conversion of DBP by liver esterase enzymes. Phthalates undergo further metabolism e.g. -COOH group replaced by –glucouronide *via* β-glucuronidase.

The major route of phthalate ester elimination from the body is urinary excretion (Kluwe, 1982). Clearance from the body is rapid. It has been determined that >90% of up to 2310mg/kg of DBP can be excreted within 48h of administration, via the urine, with undetectable levels after 5 days (Kluwe, 1982). MBP and the other metabolites are excreted in the urine mainly as glucuronide conjugates e.g. MBP-glucuronide (Foster et al., 1983; Tanaka et al., 1978). Additional metabolic products include phthalic acid (only the liver can hydrolyse phthalates to phthalic esters to phthalic acid) but the monoester (e.g. MBP) is believed to be the agent responsible for the toxic effects seen following oral DBP exposure in vivo (Blount et al., 2000b). The route of administration does not obviously affect their metabolism. DEHP is extensively metabolized by mammals, but different species show dramatic differences in their metabolite distributions. Studies in rats led to the suggestion that the enzymatic processes normally associated with oxidation of fatty acids could account for the known metabolites of DEHP found in the urine (Albro et al., 1983). In a study in which ^{14}C labelled DEHP was intravenously administered to African green monkeys, ~80% of the urinary metabolites were excreted in the form of glucuronide conjugates. This is analogous to what has been reported for the urinary metabolites of DEHP from humans, but in clear contrast to the metabolites found in rat urine. Rat urinary metabolites of DEHP are

excreted unconjugated, and consist primarily of derivatives more highly oxidized than the major metabolites produced by monkey or human (Albro et al., 1981). Additionally, traces of the treatment were still evident in the monkeys livers several months after cessation of treatment but in contrast in the rat, radioactivity was below the level of detection after three weeks recovery.

Species differences are postulated to involve kinetics of several biochemical and physiological events acting in concert or competition (Albro, 1986). However, it has been demonstrated that humans and rats share similar gut lipase activity, such that it could be reasonably expected that human intestinal metabolism of DBP would be similar to that by rats (Lake et al., 1977) . It is undetermined which metabolite plays the causal role in which biological effects, and what factors (e.g. endocrine balance) can modify the metabolism of phthalate esters and in what way.

1.5.2 Toxic effects

The human health effects of phthalates are not well understood but evidence from animal studies has raised concerns that they may affect reproduction and early fetal development, particularly in males. There are many different phthalates with slightly different chemical properties, hence their varied industrial uses and potential toxicity. The biologic activity of phthalates on hepatic peroxisome proliferation will not be covered in this review, which will focus on the effects of phthalate exposure on the male reproductive tract, especially the testis. For example, DEHP is a well-known Sertoli cell toxicant (Dalgaard et al., 2000; David et al., 2000a; David et al., 2000b; Mann et al., 1985; Oishi, 1986).

Industrial chemicals are subjected to routine regulatory toxicity testing prior to their use but the protocols are not designed to investigate the endocrine disrupting potential of synthetic compounds. Plasticizers are among the highest-volume chemicals produced, with an estimated 2–8 million tons per annum (WHO, 1992). Environmental contamination or exposure occurs indirectly as the result of the manufacture, use, or disposal by leaching out of plastic products or directly through one of the numerous consumer products (cosmetics, pharmaceuticals, etc.). DBP has extensive use in industry in such products as plastic (PVC) piping, various varnishes and lacquers, safety glass, nail polishes, paper coatings, dental materials, pharmaceuticals, and plastic food wrap. Associated with this extensive worldwide use is the high potential for human exposure to DBP in the workplace and the home environment through direct sources as well as indirectly, through contamination of water, air, and foodstuffs (Marsman, 1995).

1.5.2.1 Routine toxicological assessment

Due to concern over the potential for pervasive exposure of humans to DBP, various studies examined rats and mice after exposure to phthalates via different dose routes, at different ages, different doses and different durations. Additional studies examined the effects on rats of combining perinatal and adult subchronic exposure. Reproductive assessment by continuous breeding (including crossover mating trials and offspring assessment) and genetic toxicity studies were also conducted (Marsman, 1995).

Routine regulatory developmental toxicity tests did not induce any reproductive malformations when animals were exposed to the phthalate DEHP (Narotsky and Kavlock, 1995; Tyl et al., 1988). Toxic effects were limited to decreases in maternal food and water consumption with consequent decrease in bodyweight of dams and fetuses seen in rats. No external, visceral, and skeletal malformations or variations were noted in the DEHP exposed rat fetuses compared to the controls (Tyl et al., 1988). Then Kavlock et al (2002) published an expert panel report on the reproductive and developmental toxicity of DBP. In it, the general toxicology and biological parameters of DBP were summarised. The oral LD_{50} in rats was reported as >8000mgDBP/kg bodyweight but the majority of repeat dose studies tested relatively high doses of DBP (500 mg/kg/day) still at concentrations not normally found in the environment (maximum estimate = <5 μg DBP/kg/day) (Kavlock et al., 2002). Pregnant rats exposed to 500 mg DBP/kg/day from e12 to e21 induced a high incidence of testicular maldescent and impaired differentiation of the Wolffian duct and the prostate and external genitalia in their male offspring (Mylchreest and Foster, 2000; Mylchreest et al., 1999). The weights of the androgen-dependent organs (testes, epididymides, seminal vesicles, and prostate) were also reduced at d100 postnatal, long after cessation of treatment. Phthalate-mediated male reproductive toxicity in the developing testis is thought to occur by a complex interaction between Sertoli, Leydig, and germ cells associated with decreased fetal androgen production (Mylchreest et al., 2002). Further information regarding the toxicity related to DBP is incorporated into Chapter 3.

1.5.2.2 Human exposure

For decades, phthalates have built up to become ubiquitous pollutants and are only now being recognised as a contributor to an enormous ecological problem that is poorly understood. It was estimated that >600 000 workers in the United States are exposed to DEHP and that over 100 000 are exposed to phthalic anhydride, yet few data were available on levels of phthalates in biological fluids of these workers. One study of urine samples obtained from 48 workers in jobs with high exposure to phthalates and from 47 workers in jobs with low exposure, showed a two-fold increase in urinary phthalate concentration over

their shift, with post-shift phthalate levels of >10 nmol/ml observed in 25% of the exposed operators (Liss et al., 1985).

DBP has been found in the sediment, water, air, biota, and even in stored foods (Aurela et al., 1999; Giam et al., 1978). Biomonitoring of phthalate levels in the urine of Americans revealed DBP to be the most common phthalate ester with reprotoxic potential, that was measured in the urine of American women of child bearing age (Blount et al., 2000b). The source of the DBP peak in this sub-population was hypothesised to be associated with increased DBP exposure through their environment, given that DBP is found in cosmetics and personal care products.

Epidemiological studies have attempted to correlate exposure to phthalate-containing materials with decline in semen quality and increased risk of testicular cancer (Murature et al., 1987). A weak negative correlation between sperm density and phthalate concentration was found in a random population of college students (Murature et al., 1987), while an increase in risk for developing testicular seminomas was determined in workers occupationally exposed to phthalate-containing materials (Hardell et al., 1997). However, another study using a larger population found no increased risk for testicular cancer in these workers (Hansen, 1999).

Amann (1982) observed that in utero, adolescent, or postpubertal exposure of rats to DBP decreased the percentage of morphologically normal sperm in the ejaculate and increased seminiferous epithelial loss, and that exposure to DBP in utero or during adolescence decreased the number of sperm in the ejaculate by 23 and 20%. Human males have low reproductive efficiency; therefore, the human testis would be more vulnerable to toxicological insult, assuming similar responses occur (Amman, 1982).

The 2002 report by the American National Toxicology Programme Centre for the Evaluation of Risks to Human Reproduction (NTP CERHR) summarised various sources' data on DBP exposure (Kavlock et al., 2002). Estimated doses (μg/kg/bodyweight/day) depended on the exposure route: ambient air = 0.00026-0.0009, drinking water = 0.005-0.02, food = 0.007-7. Data was included from Canada that estimated that 0.5-11 year old children had the highest levels of estimated DBP exposure (4.3-5.0 μg/kg/bodyweight/day) of people under 19 years old, compared to exposure of people aged between 20-70 years (1.9 μg/kg/bodyweight/day), mostly via food for all ages. UK data estimated mean DBP exposure levels to be <0.48 μg/kg/bodyweight/day (1993, based on fatty food intake).

Furthermore, phthalates with similar toxicological effects may have a cumulative effect that would be expected to be additive. This body burden may be highest in certain populations, such as infants that mouth PVC- toys/ teethers, teenagers with increased food intake/kg

bodyweight and women of child bearing potential that use cosmetics and personal care products (Koch et al. 2003). For the curious, there is even a website dedicated to phthalates, albeit run by the relevant industrialists (i.e. the Phthalate Esters Panel of the American Chemistry Council): www.phthalates.org.

1.6 Aims of this thesis

There is growing evidence that male reproductive function is declining in human and some wildlife populations. This is coincident with the increasing use and prevalence of man-made chemicals in the environment over the last fifty years. One chemical, DBP, is a known endocrine disruptor that does not extensively bioaccumulate in most environmental strata, but it is continuously being released into the environment where it may act synergistically or additively with many other contaminants via an unknown toxic mechanism. In order to elucidate how DBP exerts its adverse effects, further studies of its mechanism of action and the consequences of exposure to phthalates in fetal development were necessary. This can best be achieved using a combined approach whereby animal models are used in combination with in vitro human studies (Murray et al., 2001). The primary aim of the experimental work described in this thesis was to investigate the effects of DBP on the developing testis. Pregnant rats were treated with DBP and/or MBP to assess their potential to induce a syndrome of changes in testis development in the male fetuses that parallel the human testicular dysgenesis syndrome (TDS) (Chapters 3 and 6). If successful, this model could assist in the elucidation of the causes/pathways of TDS. Human fetal testes were also exposed to DBP and/or MBP, in vitro via a novel organotypic culture system (Chapter 5).

This was a unique opportunity to investigate whether changes in the development of human fetal testes could be adversely affected by exposure to these chemicals under controlled conditions i.e. through in vitro experiments. These studies were performed in parallel with experiments exposing fetal rat testes to DBP and/or MBP in vitro (Chapter 4). An overall discussion of the role of these experiments in contributing to the elucidation of the mechanism of toxicity induced by DBP and current understanding of how this environmental contaminant might be behind the increasing levels of TDS, are described at the end of this thesis in Chapter 7.

2 General Methods and Materials

The aim of this project was to investigate the changes induced by exposure to certain phthalate esters to the fetal rat and human testis during embryonic development. This involved the use of rats for *in vivo* experiments and for the provision of fetal testes for *in vitro* experiments. Additional *in vitro* studies were performed using fetal testes obtained from aborted human fetuses. Details on the sourcing of the biological tissues, their handling and their experimental processing are provided in this chapter. Where specific procedures were used, further information is provided in the relevant experimental chapter.

2.1 Animal Work

All studies involving rats were carried out in accordance with the Animal (Scientific Procedures) Act 1986. Experienced and licensed handlers working under the relevant Home Office project and personal licenses performed the necessary procedures. The animals were housed in specific facilities provided by the University of Edinburgh. The physical location and management of these facilities changed midway through this project but this had no impact on the present studies.

The day-to-day husbandry of these animals and the majority of the licensed procedures were carried out by Mark Fisken and Denis Doogan of the MRC and Keith Chalmers of the University of Edinburgh. Huge thanks are due to them for their expert assistance. As a personal license holder, I was able to perform some procedures myself (PIL 60/8817).

All procedures were carried out according to the UK Home Office regulations, under project license 60-2442 until 2003 when it was transferred to project license 60-3045.

The background work to this project and the animal studies presented in this thesis were all carried out using the Wistar strain of rats. The colony was bred in-house (University of Edinburgh, UK) but was initially sourced from Charles River UK, a Home Office designated breeder. Latterly the colony was re-derived and re-housed in the BRF, University of Edinburgh.

2.1.1 Welfare conditions

Animals were accommodated in specially built facilities in which food (Type 3, soy free, rat, SDS diets, UK) and fresh mains tap water were available *ad libitum*. Light was provided for 12h every day from 7am until 7pm. Humidity was generally kept at 55% and the temperature generally ranged between 20 –25^{o}C.

Rats lived in cages with clear sides and solid bottoms, except during mating when grid bottomed cages were used to enable copulatory plugs to be detected on trays placed beneath the cage. Normally female rats were group housed with up to six adult females per cage with

a bedding mixture of corn cob and wood shavings. Each cage usually contained a cardboard igloo or a cardboard tunnel for environmental enrichment. All bedding *etc* was supplied by BS&S, Scotland.

For experiments that extended beyond fetal development, dams were individually housed from late gestation throughout parturition until weaning of the pups after 21 days old. Stud male rats were kept singly housed except during mating.

2.1.2 Time-mating

Female rats were time-mated so that an accurate calculation of their stage of gestation could be determined. One male and one female rat were paired in a grid-bottomed cage towards the end of the working day (~ 4pm). The next morning, the tray beneath the cage was examined for copulatory plugs. Positive detection of these plugs implied the animals had mated. The date was noted and designated embryonic day 0.5 (e0.5). Daily tray checks were continued until plugs were detected for each female. This usually took up to 5 days, reflecting the 4-5 day menstrual cycle of the female rat, of which only one day sees the dam in the oestrus phase and receptive to mating. Once a positive mating sign was detected, the female was removed from the male rat.

Stud males were derived from the in-house colony and were between 6-12 months of age at the time of mating. Dams were at least 10 weeks old at mating. Where possible, proven sires and dams were used. The standardisation of husbandry practises helped to maximise the reproducibility of the experimental work.

2.2 *In vivo* treatments and test compounds

Pregnant adult female rats were dosed once daily by oral gavage using a 10-12cm long 15-16G commercial blunt ended steel gavage cannula (Medicut, Sherwood Medical Industries Ltd., UK). This procedure caused minimal discomfort and was a Home Office recognised dosing route and placed an accurate volume of the test substance directly into the rats' stomach. The measured volume was transferred *via* attached disposable plastic 1ml insulin syringes (B-D Plastipak). Individual animals were weighed immediately prior to dosing to enable accurate individual bodyweight related volumes to be administered. The clinical signs of treated rats were checked frequently following administration to ensure that any treatment related changes in their welfare were addressed, such as accidental gavage misdosing into the lungs.

Only two different chemicals were used for treatments *in vivo*: Di-*n*-butyl phthalate (DBP) and its metabolite Monobutyl phthalate (MBP), with their respective vehicle controls.

2.2.1 Di-*n*-butyl phthalate (DBP)

CAS No.: 84-74-2, a viscous pale yellow oil, stored at rtp (Sigma D-2270, >99% pure).

For most of the studies described in this thesis, the dose of DBP administered to pregnant rats was 500mg/kg bodyweight. At this level, it has been demonstrated that there are no adverse toxicological effects on the dam but there was high reproducibility of reproductive tract maldevelopment in the male fetus without an increase in embryonic lethality (Ema et al., 1997; Ema et al., 1998). This dose level was required to induce the full syndrome of effects seen in the background work to this project.

In order to generate the dose level used, pure DBP was diluted in pure corn oil (supermarket bought), as it was lipid soluble. The use of corn oil as a vehicle is consistent with previous studies of in vivo dosing of DBP by this and other laboratories. The preparation assumed both corn oil and the DBP had similar density: 1g/ml. For example: to dose at 500mg/kg bodyweight, 5ml of DBP was made up to 10ml with corn oil, and administered at a volume of 1ml/kg bodyweight.

Treatments were freshly prepared for each experiment and were stored at room temperature between administrations, with any excess disposed of appropriately at the end of the treatment phase. The treatment regimes used are summarised in the table below.

Dose Level (ml DBP/kg bodyweight)	Treatment age range	Kill Age
500	e13.5-20.5	e21.5
500	e19.5-20.5	e21.5
Dose Level (ml corn oil/ kg bodyweight)	**Treatment age range**	**Kill Age**
1	e13.5-20.5	e21.5
1	e19.5-20.5	e21.5

Table 2.1: DBP or corn oil was administered by oral gavage, once daily, according to the tabulated regimes. All doses were given at 1ml/kg bodyweight. Necropsy was undertaken 24h after the final dose. e = the timed day of embryonic development (gestation) the treatments were given.

Separate corn oil only treated animals received pure corn oil at 1ml/kg bodyweight according to the same dosing regime as the DBP treated animals. The only anticipated vehicle effect was on bodyweight due to the calorific value of the oil.

2.2.2 Monobutyl Phthalate (MBP)

CAS No.: 131-70-4, a white powder, stored at 4-8°C (TCI Europe: P1132).

MBP is the major metabolite of DBP and generally thought to be the active agent that induces the toxicological effects on the testis following DBP exposure *in utero* (Imajima et al., 2001; Saillenfait et al., 2001; Shono and Suita, 2000).

An equivalent dose to that used in the DBP studies was used (500mg/kg bodyweight). MBP powder was slightly more water soluble, but less lipid soluble than DBP so would not dissolve in corn oil but dissolved in the organic solvent dimethylsulfoxide (DMSO, Sigma, D-2650) at a ratio of 1:0.6 w/v. DMSO is a low molecular weight compound that is liquid at room temperature and is often used as a solvent for hydrophobic compounds (Akingbemi et al., 2000). DMSO has been shown to be non-toxic at dietary concentrations as high as 1.0% (Dresser et al., 1992). The preparation assumed both corn oil and the MBP/DMSO solution had comparable densities. For example: to dose at 500mg/kg bodyweight, 5.00g MBP required 3ml DMSO to go into solution, made up to 10ml with pure corn oil, administered at a volume of 1ml/kg bodyweight. Treatments were freshly prepared for each experiment and were stored at room temperature between administrations, with any excess disposed of appropriately at the end of the treatment phase. The treatment regimes used are summarised in the table below.

Dose Level (mg MBP/kg bodyweight)	Treatment age range	Kill Age
500	e19.5-20.5	e21.5
185	e19.5-20.5	e21.5
Dose Level (ml vehicle/ kg bodyweight)	Treatment age range	Kill Age
1	e19.5-20.5	e21.5

Table 2.2: MBP was administered by oral gavage, once daily, according to the tabulated regimes. All doses were given at 1ml/kg bodyweight. Necropsy was undertaken 24h after the final dose. e = the timed day of embryonic development (gestation) the treatments were given.

The dosing levels were chosen to investigate the potency of the effects of MBP in the fetus compared to DBP and compared to *in vitro* MBP exposure. The separate DMSO control animals received a DMSO/corn oil mix according to the same dosing regime as the MBP treated animals. The maximum DMSO exposure level was 0.3ml/kg bodyweight. As with the DBP-treated animals, the only anticipated vehicle effect was on bodyweight due to the calorific value of the oil, equally affecting treated and control animals.

2.2.3 5-Bromo-2'deoxyuridine-5'-monophosphate (BrdU)

CAS No.: 59-14-3, a white powder, stored at –20°C(Sigma B-9285).

BrdU is a synthetic chemical used as a marker of cell proliferation. It infiltrates proliferating cells where it replaces the thymidine nucleotide during mitosis with an analogue base for uridine. This uridine containing DNA (U-DNA) is not corrected by the DNA polymerase quality control mechanisms in the cell, so can later be detected in the nucleus of a recently divided cell by a specific anti-BrdU antibody. Cells undergoing DNA repair also incorporate BrdU, but such cells were considered infrequent. The number of labeled/unlabelled nuclei can be counted and the Proliferation Index (PI%) per cell type then calculated.

BrdU was administered by intraperitoneal injection at 100mg/kg bodyweight in saline (0.9% NaCl, w/v) at a dose volume of 2ml/kg bodyweight. To aid solubilisation of the BrdU, the saline was heated (by submersing a sealed aliquot under running water from the hot tap) to around 40°C before the BrdU powder was added. Solubility of BrdU appeared to be temperature sensitive, though care was taken to ensure the animals did not receive a substance warmer than body temperature. Animals were injected 1.5h prior to their scheduled kill if pregnant or 1h if non-pregnant (adult males). BrdU was administered equally to vehicle-dosed control animals and treated animals.

2.3 Necropsy Procedure

Adult animals were routinely killed by CO_2 asphyxiation followed by cervical dislocation, under Schedule 1 of the Animal (Scientific Procedures) Act 1986. In the occasional absence of CO_2, animals were killed by cervical dislocation by an experienced technician, followed by exsanguination. Following removal from the freshly killed dam, fetuses were immersed in refrigerated buffer (0.01M PBS, Sigma); late gestation fetuses (from e19.5) were decapitated prior to immersion

2.3.1 Gross Dissection

Rat fetuses were removed using an established gross dissection technique.

The abdomen of the supine dam was opened and the intact gravid uterus removed.

The umbilical cord was severed for each fetus prior to its removal from the amniotic sac and the body immersed in refrigerated 0.01M PBS. The carcasses were transported in PBS, on ice. This minimised the onset of any degenerative changes prior to fine dissection and tissue recovery.

2.3.2 Fine Dissection

Once immersed, the fetuses were relocated into the designated dissection area of the research laboratory. The fetal testes were removed under a binocular microscope with a transilluminated stage, according to the technique described below, and placed in fresh chilled PBS until processed.

The supine fetus was cradled between pieces of PBS soaked tissue for stability.

A careful horizontal incision was made beneath the liver, in line with the umbilical cord. Abdominal tissues such as intestines were gently removed to reveal the gonads and urogenital tract beneath. The gonads were located in the abdominal cavity between the kidneys and the bladder, depending on gestational age and treatment.

2.3.3 Microdissection

Retrieved testes were further trimmed of the attached reproductive duct e.g. fetal epididymides/ mesonephros. The trimming was carried out using 27G needles (Monoject, sterile needles, 0.4mm x 12mm) attached to disposable plastic 1ml insulin syringes, for improved dexterity. This was undertaken using a binocular dissecting microscope (Leica, MZ6) at up to 4x magnification. The stage had in-built underlighting but additional external cold lights (Leica CLS 150x) were also used for maximum visibility and minimal heat damage.

2.4 Tissue preservation

Retrieved tissue was preserved either by snap freezing or by fixing in Bouin's. Whole fetal testes froze instantly on dry ice in 1.5ml Nunc cryovials, were carefully labeled and archived at –80°C, until used. When weights were required, tissue was weighed just prior to use. Alternatively, testes were fixed by immersion in Bouin's solution, a formaldehyde based fixative containing picric acid. It is the preferred fixative for testis tissue. Fetal tissue was immersed in at least 10x its own volume of Bouin's for 1h at room temperature in an airtight container and was weighed after fixation. Adult testes were weighed prior to immersion fixation in an air-tight container for 3h, cut transversely with a razor blade and the halves re-immersed for a further 3h, cut transversely again and the quarters immersion fixed for a final 2h. Following fixation in Bouin's, tissue was transferred to 70% ethanol and stored in an airtight container at room temperature until processed for paraffin embedding.

2.4.1 Fixed Tissue Processing

After the ethanol transfer, tissue was processed through a series of graded alcohols using the 18h automated cycle on a Leica TP-1050 (Leica UK Limited, UK) tissue processor and

finally embedded in molten paraffin wax by hand. The cooled wax block was stored at room temperature until needed. Efforts were made to deliberately orient the processed testes prior to embedding to try to standardise the plane of sectioning generated (see section 2.6.1.1).

2.5 *In vitro* experiments

Fetal testes from untreated rats and aborted human fetuses were recovered then further dissected to produce explants suitable for *in vitro* culture experiments. A culture method was developed by adjusting/ combining two in-house methods: one based on the experiments of Robinson *et al* (2003) for the fetal human ovary and testis and the other established by Thomson *et al* (1997) for the fetal rat prostate (Robinson et al., 2003; Thomson, 1997). The final method was achieved after much fine-tuning and is detailed in Chapter 4, but the principles are outlined below.

2.5.1 Explant preparation from the rat fetal testis

One of the key aspects of maintaining viability of tissue in culture, was ensuring sufficient oxygen and nutrient supply to the whole of the explant. The process of dissection disrupted the normal distribution system by disturbing the blood supply to and through the tissue. In order for nutrients to reach the tissue, the explants needed to be surrounded in a bath of nutrient rich media so that the vital components could reach their target by diffusion. Diffusion is a passive process; consequently its effects are not far reaching. To minimise the possibility of tissue dying through oxygen or nutrient depletion, the testes were carefully sliced into thin sections.

However, as the fetal testis is an actively and rapidly developing tissue, it was necessary to minimise the level of developmental disruption the explant preparation caused. It was known that some of the cells in the fetal testis arrive by migration from the adjacent mesonephros and coelomic epithelia tissues (see Chapter 1). Though the majority of migration was considered complete by the sampling ages used (generally e19.5), there may have been residual migratory cells in the organ periphery, particularly with younger samples as normal migration has been proposed to continue until e18.5 in the rat (Tilmann and Capel, 2002). It was considered critical to ensure that every explant included a portion of the surrounding tissues, by using a specific plane of dissection to generate the explants (Figure 2.1). The effect of fetal age on the number of explants generated is described in Chapter 4.

To achieve these fine slices, a scalpel blade (Product no. 0201, Swann Morton, UK) was used to make neat incisions through the testis along the target plane. A single incision was made to reduce shearing injuries to the tissue. The tissue was steadied with a 23G needle

attached to a disposable plastic 1ml insulin syringe. Originally, acupuncture needles were used (Acumedic Ltd, 30mm x 0.25) but they proved too flexible.

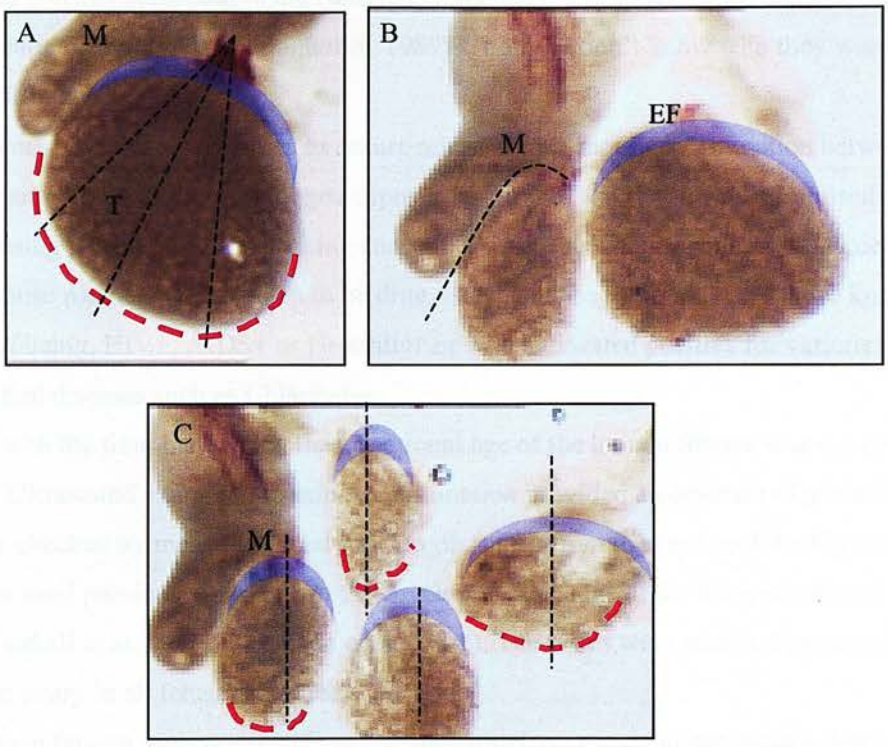

Figure 2.1: These 3 panels show the step-wise dissection of an e20.5 fetal rat testis into explants suitable for *in vitro* maintenance. Panel A shows an intact testis (T) with the adjacent mesonephros (M) still attached. The vasculature of the attached tissue was still clearly visible. The coelomic epithelium (- - - -) and rete regions (blue crescents), involved in cell proliferation/ migration, are highlighted. The lines of dissection are also shown (----). Panel B shows a midway point with the dissection almost completed in Panel C, the quartered explants still required a final vertical dissection to generate 8 explants per testis. The efferent ducts (EF) just needed to be trimmed away from the first explant.

2.5.2 Explant preparation from the human fetal testis

Where appropriate, the recovered fetal human testes were handled as similarly as possible to the rat tissue.

2.5.2.1 Acquisition of human tissue

Human fetal testes were retrieved from medical terminations of pregnancy between 14-20 weeks of gestation (14-20w/40w). Termination of pregnancy was induced in the Simpson's Maternity Hospital, Edinburgh as previously described (Gaskell et al., 2004; Robinson et al., 2001). None of the terminations were for reasons of fetal abnormality, and all fetuses selected for study appeared morphologically normal. The necessary ethical permission was

granted (Lothian Pediatrics/ Reproductive Medicine Ethics Subcommittee) to Professor Richard Anderson at the MRC Human Reproductive Sciences Unit, Edinburgh. Relevant informed consent according to the National Guidelines, was sought from and granted by the female patients involved (Polkinghorne, 1989). Though I don't know who they were, I am indebted to them.

At all times, great care was taken to ensure minimal transfer of contamination between the handler and the fetal tissue. Pathogen exposure from the fetal tissue was minimised further by screening patients, prior to seeking consent, for high risk of contamination. Patients or those whose partners were known to be drug users, were excluded as were those known to be at risk of being, HIV+, AIDS+ or Hepatitis+ or who had tested positive for various sexually transmitted diseases such as Chlamydia.

Unlike with the time-mated rats, the gestational age of the human fetuses was not accurately known. Ultrasound examination prior to termination provided an estimate of gestational age, this was checked by measuring fetal foot length (mm), from heel to tip of the big toe. This has been used previously to confirm the developmental stage of the fetus (Anderson et al., 2002; Gaskell et al., 2004; Robinson et al., 2001). The testes were readily distinguishable from the ovary in all fetuses sampled.

The human fetuses were non-viable at the time of delivery and did not require death to be caused or confirmed. On receipt of the carcass from the hospital, it was transferred to a designated dissection suite in the research laboratory where the testes were removed for use. The remains were then returned to the hospital for appropriate disposal. All actions were carefully accounted for and archived. Sample availability was beyond the influence of the investigators.

2.5.2.2 Dissection of the human fetal testis

Human fetal testes were dissected into small explants as per the rat tissue. As the human organs were larger (>10mg) than the rat testes (~1mg), more explants could be generated per specimen (Figure 2.2). Unlike for the rat, the size of the human testes precluded the allocation of rete and coelomic epithelial regions between the explants.

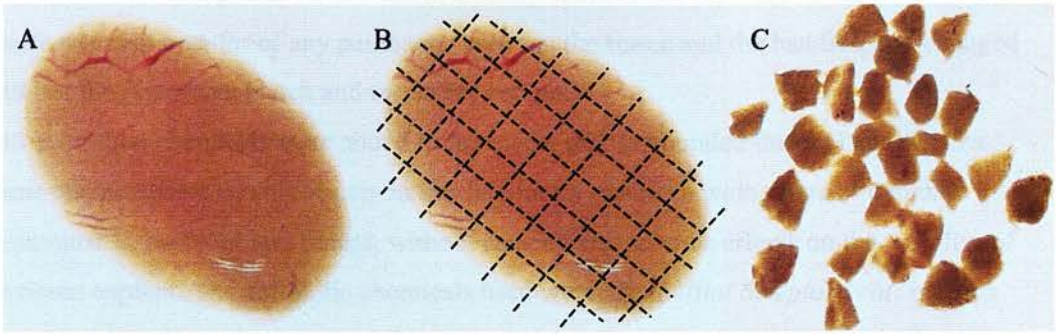

Figure 2.2: Panels illustrate the step-wise dissection of a 14/40w fetal human testis into explants suitable for *in vitro* maintenance. Panel A shows an intact testis with its distinct vasculature still clearly visible. Panel B shows the proposed lines of dissection. Panel C shows the end-result: a collection of approximately evenly sized explants, ready for incubation.

2.5.3 Primary tissue culture conditions

Cultures were incubated at 37°C, corresponding to the temperature of fetal testes *in situ*. The environment within the incubator (Gallenkamp CO_2 incubator) was humidified and maintained at 95% air/ 5% CO_2.

2.5.3.1 Culture Set-up

Once the testes were sliced into explants and the media made up to include any treatments, the culture system could be assembled. Assembly took place in a sterile environment, established in a class 2 safety cabinet. The explants were gently transferred from the holding dish in chilled PBS to the synthetic membrane of a specially designed culture well insert (Millicell 0.4μm PICM 01250, Millipore UK). The membrane was porous (0.45μm) and raised by a plastic scaffold. This insert was placed in a well of a 24 well culture plate (Corning). Pre-warmed (37°C) media was added, 200μl to the well and 200μl within the scaffold to immerse the explants nesting on the insert membrane. Explants were distributed evenly between the wells per experiment, with a maximum of 5 per insert. Up to 24 experiments could be set up per plate. This enabled replicates of both control and treated samples to be run under identical conditions.

2.5.3.2 Culture contamination

A potential problem area encountered with explant cultures was the risk of contamination with microorganisms, so practical measures were taken to minimise this possibility. It was not practicable to procure the testis explants in an entirely sterile environment, so sterility was compromised from the outset. Sterile techniques were practiced where possible. For example, fresh sterile PBS was used during each phase of dissection. All media preparation

and experiment set-up was carried out in a safety cabinet. Gloves were worn at all times to ensure minimal transfer of any pathogens between the tissue and the handler, and changed between the dissecting bench and the safety cabinet.

Anti-microbial chemicals were added to the media that surrounded the explants *in vitro*. These chemicals are used routinely in this laboratory against a wide range of common contaminants, bacterial and fungal, without causing any adverse effects on the viability of the tissue explant. The antibiotic chemicals used were: *Penicillin/ Streptomycin:* inhibits bacterial cell wall synthesis and inhibits successful prokaryote protein synthesis (P-4333 Sigma, UK) and *Amphotericin:* a toxin that in small dose is used to disrupt fungal membrane permeability, enabling small molecules to leak out (A-2942 Sigma, UK).

2.5.3.3 Culture Media

The basal media used by Robinson *et al* (2003) for human gonadal fetal explant cultures was Alpha MEM (AMEM, Gibco Cat No. 41061-029) whereas Thomson *et al* (1997) cultured fetal/ neonatal rat prostates in DMEM/F12 media (Gibco Cat No. 2104-025) (Robinson et al., 2003; Thomson, 1997). The effect of these and alternative media regimes on fetal rat testis viability *in vitro* was assessed and the results are detailed in Chapter 4 and summarised below:

	Robinson protocol	Product code	Thomson protocol	Product code
Basic Media	Alpha MEM	41061-029	DMEM/ F12	21041-025
Supplements	L-Glutamine	G-7513	Transferrin (Bovine)	11107-018
	Sodium Pyruvate	S-8636	Insulin	I-0516
	ITS	I-3146	-	-
	BSA	A-2153	-	-

Table 3: Summary of media supplements used with two different complete media regimes, used to support *in vitro* incubation of fetal testes. Note: all components were purchased from Sigma, UK, except for the basic media and Transferrin, which were from Gibco, UK.

2.5.3.4 Culture treatments

Test compounds were added in known amounts to the explants *via* the culture media. Explants were exposed to the chemicals for the entire culture period except for treatment with BrdU. Stock solutions were stored at –20°C. When required, stock solutions were diluted in culture media to give the desired concentration of test solution and stored at 4°C, except for BrdU which was freshly prepared just prior to use in warmed (37°C) media.

Equivalent amounts of the solvent were added into media for the control cultures. A summary of the compounds used, their concentrations and solvents are tabulated below:

Chemical Treatment	Source/ code	Solvent	Stock Concentration	Working Concentration
MBP (CAS No.: 131-70-4)	TCI Europe P1132	DMSO	1M	$1x10^{-10}$ M to 10^{-3} M
DBP (CAS No.: 84-74-2)	Sigma D2270	DMSO	2M	1mM
human Chorionic Gonadotrophin	Serano, USA Profasi ™	Media	10iu/ml	0.1iu/ml
22-R-hydroxycholesterol (CAS No.: 17954-98-2)	Sigma H9384	Media	1M	50μM
Ketoconazole (CAS No.: 65277-42-1)	Sigma K1003	Ethanol	1M	100μM
Follicle Stimulating Hormone	Organon, UK Puregon ™	Media	1iu/ml	50miu/ml
BrdU (CAS No.: 59-14-3)	Sigma B9285	Media	5mg/10mls	10μg/ml

Table 2.4: Summary of chemical treatments added to *in vitro* experiments.
Note: stock concentrations were typically at least 100x the concentration required in the media during the experiment (working concentration) to minimise any vehicle effects.

2.5.3.4.1 MBP and DBP

See sections 2.2.2 and 2.2.1 for more information about these chemicals. MBP is more hydrophilic than its parent compound (DBP) but neither are easily water-soluble. To this end, the compounds were first dissolved in DMSO before being diluted into media to make the target working concentration for the *in vitro* experiments. This preparation method also kept the compounds in solution.

It had been reported that at levels of >1%, DMSO has been shown to result in a variety of cellular metabolic changes (Stocco et al., 1995) but at <0.1% it does not significantly affect Leydig cell testosterone production *in vitro* (Akingbemi et al., 2000). If DMSO did have an inhibitory effect on steroidogenesis, this would be highlighted in the DMSO control samples, but this did not prove to be the case.

2.5.3.4.2 human Chorionic Gonadotrophin (hCG)

Chorionic Gonadotrophin is a glycoprotein hormone produced by trophoblastic cells of the developing placenta and has been used clinically to address androgen deficiency presenting as cryptorchidism (Kiely, 1994). It binds to the cell surface receptor for luteinising hormone

(LH-R), where it acts as a "super agonist" initiating a cascade of responses including activating adenylyl cyclase and stimulating the concentrations of the second messenger cAMP which in turn mediates a multitude of intracellular activities. In the testis, it binds to the LH-R on Leydig cells, inducing an increase in the conversion of cholesterol to testosterone. In these experiments, hCG is exploited for its 'super agonist" properties on the LH-R, exciting a greater level of testosterone production than equivalent amounts of LH (Zhou and Hutson, 1995). hCG is not normally expressed by the rat placenta so the commercially available product used in these experiments is human derived, hence hCG and (Habert and Picon, 1990). hCG concentration was used here at 0.1iu/ml, as reported, but can be measured as g/ml which can be converted to international units (iu) using the following scale: 1mg = 10 000iu (Huhtaniemi et al., 1977)

2.5.3.4.3 22-(R)-Hydroxycholesterol (22-R-CHO)

One of the metabolic intermediates in the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage P450 cytochrome is 22-(R)-Hydroxycholesterol (Burstein, 1976; Burstein, 1975). As pregnenolone is the first steroid intermediate for all steroid hormones, the rate at which it is formed determines the biosynthetic rate of overall steroid production (Papadopoulos et al., 1991). By providing the testis explants with an excess of this intermediate, 50μM, the rate limiting step is "skipped over", inducing a dramatic increase in steroid biosynthesis downstream of the rate-limiting step, measured as testosterone production (Akingbemi et al., 2000). It has no clinical applications.

2.5.3.4.4 Ketoconazole (KTZ)

Ketoconazole is used primarily as an anti-fungal agent due to its ability to inhibit the synthesis of fungal sterols (Pont A, 1982). It is also used to control endocrinopathies in humans (Gray et al., 1999). In mammals, KTZ competitively inhibits NADPH –cytochrome P450 dependent enzymes (Higashi Y, 1987). In the testis, steroidogenesis is inhibited due to the various cytochrome P450 enzymes including $P450_{scc}$, 17α-hydroxylase ($P450_{c17}$) and 17,20-lyase ($P450_{c17\text{-}20}$) (Miossec et al., 1997). The anti-androgenic action of KTZ means it has been used therapeutically for the treatment of prostate carcinoma. In these studies it was selected to demonstrate inhibition of fetal testis testosterone production *in vitro* at 100μM, based on previous experiments in this laboratory (PhD thesis, Qureshi, 1992).

2.5.3.4.5 Follicle Stimulating Hormone (FSH)

FSH is produced in the gonadotroph cells of the anterior pituitary, positioned below the brain, after stimulation by Gonadotrophin Releasing Hormone (GnRH) from the hypothalamus. FSH targets the Sertoli cells primarily and their inhibin production, though it

can (indirectly) influence Leydig cell androgen production levels too. Inhibin-B secretion by Sertoli cells is part of a complex negative feedback mechanism that suppresses FSH secretion by the pituitary. FSH secretion affects final testis size through the regulation of Sertoli cell proliferation in the perinatal/ prepubertal period and paracrine regulation of Leydig cell steroidogenesis (Markkula, 1996; Matikainen et al., 1994). In these studies, FSH was used at 50mIU/ml to investigate whether Sertoli cells in the explants were sensitive to stimulation.

2.5.3.4.6 5-Bromo-2'deoxyuridine-5'-monophosphate (BrdU)

See section 2.2.3 for more details. BrdU is a synthetic chemical used as a marker of cell proliferation. It was added to warm media for the last 4h of the *in vitro* experiments with rat testis explants but the last 6-8h of the *in vitro* experiments with human testis explants.

2.5.3.5 Tissue Handling

At the end of the culture period, media was removed and stored at –20°C until assayed and the tissue explants were carefully removed from the insert and processed as described for *in vivo* treated tissue (see section 2.4). Due to their small size, explants were fixed for only 30 mins in Bouin's before transfer to 70% ethanol.

2.6 Protein Investigations

2.6.1 Immunohistochemistry

Antibodies were used to highlight the location of proteins of interest within fixed tissue. All immunostaining, or its absence was reviewed in the context of the tissue morphology and treatment, alongside proper experimental control tissue. The principles applied and the protocols followed are outlined below.

- sectioning of fixed tissue and mounting of sections onto glass slides
- dewaxing and rehydration of sections
- retrieval of the target antigen for detection by the specific primary antibody
- blocking non-specific antigens
- incubation of tissue with specific primary antibody
- detection of the primary antibody by an amplification system
- visualisation of the amplified binding = staining
- counterstaining of non-stained tissue
- storage of stained sections for reference

2.6.1.1 Sectioning

Wax blocks containing processed fixed tissue, were cooled from room temperature prior to being cut. Cooling made the wax more rigid and easier to slice. 5μm sections were cut using a Microtome (Leica, model RM 2135). The sections were floated in a water bath at 45-50°C (Lamb RA, model E/65) to smoothen out any wrinkles in the cooled wax/ tissue by re-

heating it gently. The heated sections were then mounted onto electrostatically charged glass slides (BDH, Cat No.: 406/0179/00) and stacked in a metal rack. The slides were dried for at least 3h at 50-60°C, usually overnight (Lamb RA, model E28.5). Once dry, the slides and tissue could be stored in a dust free room temperature environment indefinitely or until used.

2.6.1.2 Dewaxing and re-hydration

Protective wax was removed from the tissue by immersing the slide in Xylene for 5 mins at room temperature, x2. Longer immersion was occasionally required. Then the tissue was rehydrated by immersion in absolute alcohol (AA) for 20s x2, 95% alcohol for 20s, 70% alcohol for 20s and then rinsed in water.

2.6.1.3 Antigen retrieval

Fixatives such as Bouin's work by inducing intra-molecular cross-linkages. These can mask target proteins (antigens) and thus prevent their immunodetection. Antigen retrieval describes the process by which the cross-linkages are partially undone by the application of heat and change in pH. It was not required for all immunodetection protocols.

Re-hydrated tissue was boiled in buffer. Different antigens required different buffers. Retrieval took place in a commercially available domestic pressure cooker (Tefal, Clypso), in which the slides were immersed in 2L of heated buffer and boiled under pressure for a timed period, usually 5 mins. The slides were then left in the buffer as it cooled, typically 20 mins. Once cool enough to handle, the slides were rinsed in cold tap water.

Buffer	Molarity	pH
Citrate	0.01M	6
Glycine/ 0.1%EDTA	0.05M	8
Glycine/ 0.1%EDTA	0.05M	3.5

Table 2.5 Summary of buffers used for antigen retrieval. Citrate buffer was the most commonly used.

2.6.1.4 Blocking

Molecules other than the target antigen are revealed in these processes and may compromise the specificity of antibody binding. This might be due to their affinity for the primary or secondary antibody or even the chemicals used to visualise the antibody binding. This undesirable binding was blocked deliberately before the primary antibodies were added. Slides were washed between various stages.

Peroxidase block: Endogenous peroxidase activity was quenched by the immersion of the retrieved slides in a solution of methanol and 3% hydrogen peroxide (H_2O_2) for 30 mins at room temperature (30% H_2O_2, BDH Cat No.: 101284N). Without this step, there was a

risk of these enzymes compromising the horseradish peroxidase step of amplification (see 2.6.1.6.1).

Serum block: Non-specific binding of components in the secondary antibody was minimised by the application of a dilute solution of serum from the species in which the secondary antibody was raised. For example swine serum was used for 30 mins at room temperature to block tissue due to be exposed to a swine raised anti –rabbit secondary antibody.

Avidin/ Biotin block: Human tissues were prone to endogenous avidin or biotin expression. This was minimised by incubating the tissue with avidin for 30 mins at room temperature, followed by 2 washes in buffer (see below) and incubation of the tissue in a pool of biotin in washing buffer for 15 mins at room temperature. A kit containing the avidin/biotin was used (Vector, SP-2001). Endogenous expression would interfere with later steps of the imunohistochemistry (see 2.6.1.6.1).

Washing Buffer: Where necessary, slides were washed by immersion in 0.05M Tris buffered saline at pH7.4 (TBS). This was diluted as required from a 0.5M stock. Slides were rocked (20-40rpm) for 2 x 5 mins with fresh buffer at room temperature.

2.6.1.5 Primary Antibodies

Following antigen retrieval and blocking, slides were washed then removed from TBS and the area around the tissue was carefully dried to remove excess buffer. Care was taken at all stages to avoid wiping the tissue off the slide but to keep the tissue hydrated. Sometimes it was helpful to draw a perimeter around the tissue with a diamond pen on the underside of the slide to highlight the location of small pieces or on the tissue side with a PAP pen to create a waterproof perimeter that held the pool of solution over the tissue during incubation periods. Blocking serum was added to slides maintained in a humidity chamber at room temperature, for 30 minutes. This was replaced by a solution of primary antibody in blocking serum at an optimised concentration. Typically the slides were left to incubate in the humidity chamber overnight at 4°C. The exact conditions (concentration/ temperature/ duration) were optimised for each primary antibody used. The table below summarises the general conditions used.

Target Antigen	Source	Concentration	Host Species	Antigen Retrieval	Exposure duration/ temperature
AMH (Anti-Müllerian Hormone)	Santa Cruz (sc-6886)	1:1000	Goat	None	Overnight, 4°C
AR (Androgen receptor)	Santa Cruz (sc 0816)	1:200	Rabbit	Citrate	Overnight, 4°C
3-βHSD (3-beta hydroxysteroid dehydrogenase)	Gift: Prof J I Mason, University of Edinburgh	1:4000	Rabbit	None	Overnight, 4°C or 3h, rtp
BrdU (5-Bromo-2'deoxyuridine-5'-monophosphate)	Fitzgerald Industries (20-BS17)	1:2000	Sheep	Citrate	Overnight, 4°C
Inh-α (Inhibin α- subunit)	Gift: Prof P.T.K. Saunders, MRC HRSU, Edinburgh	1:2000	Mouse	None	Overnight, 4°C
$P450_{scc}$ (Cytochrome P450 side chain cleavage)	Chemicon International (AB1244)	1:200	Rabbit	None	Overnight, 4°C
SMA (Smooth muscle actin)	Sigma (A2547)	1:2000	Mouse	None	Overnight, 4°C
SR-B1 (Scavenger receptor Protein-B1)	Novus Biologicals (NB400-104)	1:200	Rabbit	Citrate	Overnight, 4°C
WT-1 (Wilms Tumour protein)	Santa Cruz (sc-0192)	1:1000	Rabbit	Citrate	Overnight, 4°C

Table 2.5: Summary of primary antibodies used for immunohistochemistry. Santa Cruz products supplied *via* Autogen Bioclear UK Ltd, Wiltshire, UK.

2.6.1.6 Secondary Antibodies

After incubation, the primary antibody was washed off the slides (2x 5mins in TBS).

It was necessary to amplify the localisation of the primary antibody. This was done using a secondary antibody, raised against a species-specific sequence on the arm of the primary antibody. This secondary was then amplified directly or indirectly (Figure 2.3).

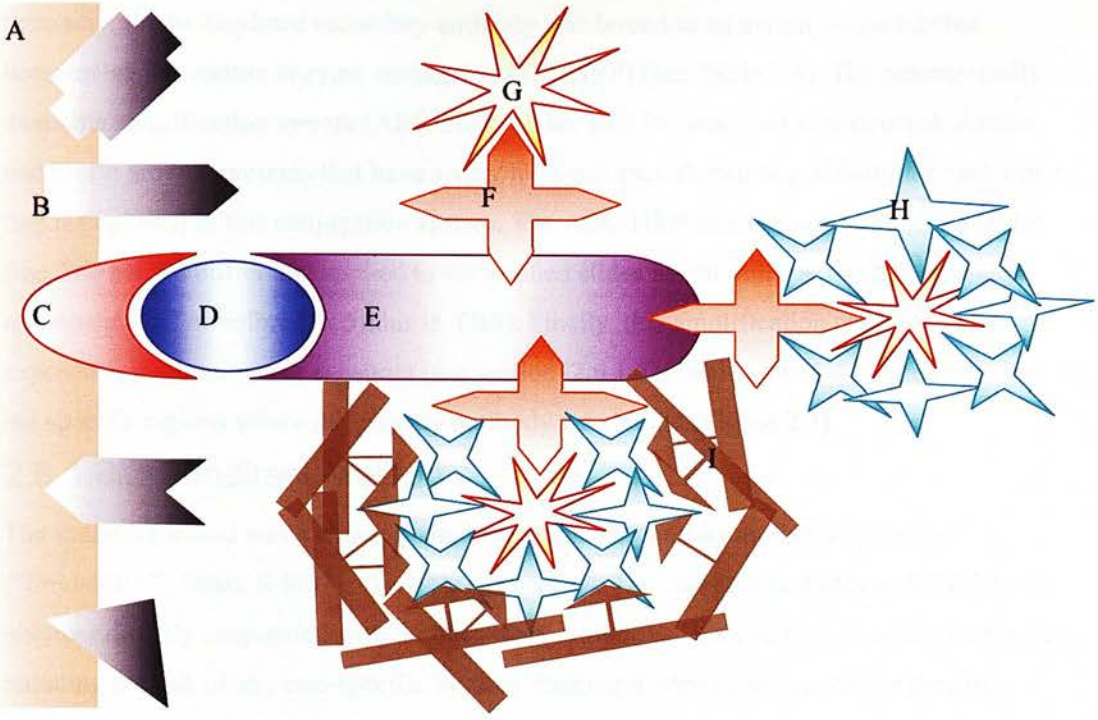

Figure 2.3: Schematic representation of the principles of the direct immunohistochemistry protocol. Within cells (A), proteins are expressed (B) of which particular parts are recognised as antigens (C) by specific primary antibodies (D), which then bind to them. A secondary antibody (E) with its biotin conjugates (F) binds to species-specific regions on the primary antibody. An avidin/biotin/ enzyme complex (G) associates itself with the conjugated biotin. The enzyme (H) reacts with the chromogenic substrate to create a visible colour change (I) at the site of the antigen expression.
The indirect method replaces E, F, G and H with a single intermediate complex.

2.6.1.6.1 Direct Method

The direct method was a three-step process. Firstly, the tissue was incubated with a specific primary antibody (Table 2.5), which bound with a specific biotin labeled secondary antibody (Table 2.6).

Target Antigen Species	Source/ code	Concentration	Host species
Rabbit	Dako, E0353	1:500	Swine
Sheep	Vector, BA 6000	1:500	Rabbit
Goat	Vector, BA 5000	1:500	Rabbit
Mouse	Dako, E0464	1:500	Rabbit
ABC-HRP (All secondary antibodies)	Dako, K0355	as supplied	n/a

Table 2.6: Summary of secondary antibodies used for immunohistochemistry. They all required incubation for 30mins at room temperature.

Secondly, the biotinylated secondary antibody was bound to an avidin/biotin labeled horseradish peroxidase enzyme conjugate (ABC-HRP) (see Table 2.6). The commercially available amplification system (ABC-HRP, Dako, K0355) was used as instructed. Avidin and biotin are two proteins that have a very high and specific binding affinity for each other that is exploited in this conjugation system. The ABC-HRP mixture was solubilised in salt free Tris pH 7.6 buffer and applied to the washed slides for 30 mins, at room temperature and washed off as before (2x 5mins in TBS). Finally, this amplification complex was exposed to a chromogenic substrate (see section 2.6.1.7) causing a colour change reaction in the specific regions where the primary antibody was bound (Figure 2.3).

2.6.1.6.2 Indirect Method

The indirect method was a two-step process, using a secondary antibody/ polymer ("Envision +", Dako, K4007), available per species. The complex includes an HRP-labeled polymer directly conjugated with the secondary antibody. This complex is avidin/biotin free, reducing the risk of any non-specific binding, making it very sensitive. As for the direct method, the amplification complex was exposed to a chromogenic substrate (see section 2.6.1.7) to cause a colour change reaction in the specific regions where the primary antibody was bound.

2.6.1.7 Chromogenic Detection

In order to see where the primary antibody localised a visual colour change was needed, that could be reviewed using a standard light microscope, to distinguish the specific protein expression from the rest of the tissue. The colour of the precipitate generated at the site of the original antigen, varied according to the chromogen substrate used.

Brown: *DAB (3,3 DiAminoBenzidine)*, used most routinely and was bought in as a concentrated solution (Dako, K3468) with specific buffer to be diluted just prior to use. It was added to slides after unbound ABC-HRP complex was carefully washed away (2x 5mins in TBS). Depending on antibody, it was incubated briefly from 30s to 5mins, timed per experiment. Immersing the slide in tap water stopped the completion of DAB development.

Blue: Fast Blue was used as the second stain in double staining experiments. Made up in-house, this chromogen took longer to develop than did DAB (around 5-20 mins) and produced a blue stain, similar to the haematoxylin counterstain, so they were not used together. Chromogen was made up to 1mg/ml with fast blue salt (Sigma, F3378), mixed well and passed through a 0.2μm filter just before use. The completion of development was timed per experiment and stopped by immersing the slide in tap water.

Buffer: this is made in a two-step process: 0.1M Tris (pH 8.2) = 12.1g Tris (BDH 271197K), 950ml distilled water. Make up to 1l at pH8.2 with concentrated HCl acid. Per 100mls: 20mg

Napthol AS-MX phosphate (Sigma N-5000), 2ml Dimethyl formamide (Sigma D4254). Mix together in glass tube, add 98ml 0.1M Tris (pH 8.2) buffer.

2.6.1.8 Microscopy

Photographed images were compiled using Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA).

2.6.1.8.1 Light microscopy

Nonfluorescent images were examined and photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a DCS330 digital camera (Eastman Kodak, Rochester, NY).

2.6.1.8.2 Fluorescent microscopy

Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Fluorescent chromogens were used to emphasize co-localisation as DAB could overwhelm Fast blue staining, making co-localisation of proteins difficult to interpret.

Fluorescence results when sensitive materials are irradiated by light at a specific wavelength. This excites atoms within the compound to a higher energy level, which is released and detected as visible light when they drop back down. This emitted light has a longer wavelength and lower energy than the "exciting" light that is generated by a laser beam incorporated into the microscope. To distinguish between the two types of light, the microscope is fitted with two filters, one of which enables the sample to be irradiated by light at a specific wavelength, the second separates out the excitation light from the emitted light. The colour of emitted light seen, depends on its wavelength, which varies depending on the sensitive chemical used. The fluorescent chromogens used are summarized in Table 2.7.

Fixed tissue destined to be examined using fluorescent immunohistochemistry were pre-treated as detailed in section 2.6.1 until the addition of the primary antibody, which is diluted in the appropriate normal serum/PBS/BSA then incubated overnight at 4°C as before. After washing in PBS, sections were incubated for 30min with the appropriate secondary antibody, namely one that is conjugated to HRP (Dako) diluted in normal serum/PBS/BSA. After washing in PBS, slides were incubated for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; Perkin-Elmer Life Sciences, Boston, MA) diluted 1:50 in the buffer supplied, to amplify the immunostaining and produce red fluorescence. Washing in PBS followed this and all subsequent steps. Where co-localisation of the expression of two proteins was required, slides were re-incubated for 30 min with the appropriate normal serum/PBS/BSA,

followed by overnight incubation at 4°C with the additional primary antibody diluted in the appropriate normal serum/PBS/BSA then incubated overnight at 4°C as before. After washing in PBS, sections were incubated for 30min with the appropriate secondary antibody, namely one that is a biotinylated secondary antibody (Vector Laboratories Inc., Peterborough, UK) diluted in normal serum/PBS/BSA. This was followed by a 1h incubation with streptavidin conjugated alexa 488 probe (Molecular Probes, Poort Gebouw, Holland) diluted 1:200 in PBS, producing green fluorescent immunostaining. Slides were counterstained by incubating for 2 min with a nuclear specific blue fluorescent label (To-Pro 3; Molecular Probes) diluted 1:2000 in PBS. Slides were then washed in PBS and mounted in aqueous mounting medium (Permafluor; Beckman Coulter, High Wycombe, UK) (Mahood et al., 2004).

Fluorescent complex (Supplier)	Dilution	Exposure duration/ temperature	Excitation wavelength (nm)/ emitted light colour
Tyramide Cy3 (Perkin-Elmer Life Sciences)	1:50	10min, rtp	546/ red
Streptavidin conjugated alexa 488 (Molecular Probes)	1:20	1h, rtp	488/ green
To-Pro 3 (Molecular Probes)	1:2000	2min, rtp	655/ blue

Table 2.7: Summary of fluorescent labeled complexes used.

2.6.1.9 Terminal dUTP nick end labeling (TUNEL)

This established commercially available protocol enables cells undergoing programmed cell death, apoptosis, to be highlighted by visualising their DNA fragmentation. This discerns them from cells undergoing necrosis. Apoptotic cells were detected in situ by use of the TUNEL method as described by Livera *et al.* (2000). This assay detects fragmented DNA in apoptotic cells by catalytic incorporation of fluorescein-12-dUTP at the 3'-OH ends of the DNA using the terminal deoxynucleotidyl transferase enzyme, visualised using DAB as described previously (Figure 2.4).

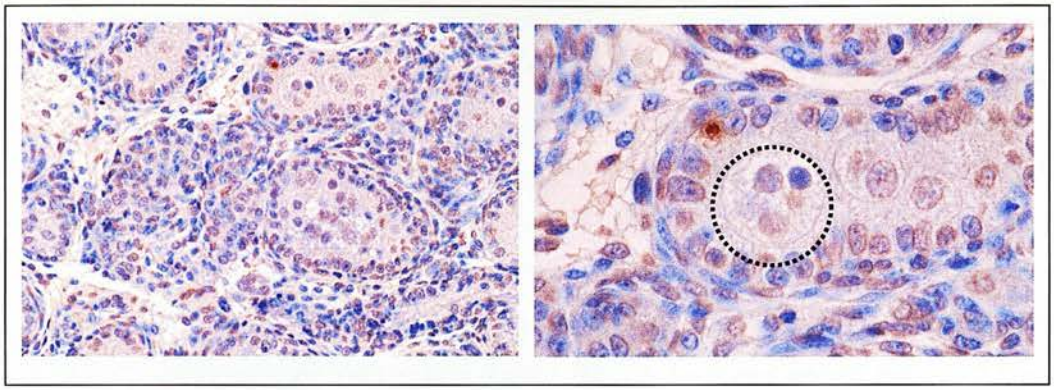

Figure 2.4: TUNEL positive staining of an intratubular cell, at the periphery of a seminiferous cord within an e21.5 rat testis. Note the absence of brown staining from the abnormally nucleated fetal germ cells at the centre of the cord (dashed circle). The panel shows a cross section through an e21.5 rat testis at x40 and x100 magnification.

Necrotic cells were recognisable microscopically by their darkly counterstained pyknotic nuclei. Ideally, all necrotic tissue would have been discarded from further analysis but due to the permanent appearance of this artifact in the centre of cultured explants, necrotic tissue was noted but investigative focus was placed on the viable peripheral tissue per sample.

2.6.1.10 Haematoxylin and Eosin Staining (H&E)

These two dyes were used as counterstains for sections following immunohistochemistry but were also used together when inspection of the tissue morphology was the only objective. These stains are routinely used in medical and veterinary histopathological studies.

Harris's haematoxylin: Stains cell nuclei. Slides were immersed for 5 mins in a bath of haematoxylin, rinsed in tap water, briefly immersed in 1% acid alcohol to remove any non-specific cytoplamsic staining and rinsed in tap water. The dye was developed by immersing the slides in Scott's tap water for 30s until the reddish stain turned blue. This was checked using a standard light microscope.

Eosin: Stains cell cytoplasm. Slides were immersed in a bath of Eosin for 30s then rinsed thoroughly in tap water. The pink colouration was checked using a standard light microscope.

Following staining, slides were removed from the bath of tap water and dehydrated before mounting with a coverslip secured with Pertex™ as described below.

2.6.1.11 Coverslips

After tissue had been counterstained, it was protected by the application of a borosilicate glass coverslip (VWR, 22x32mmx1,Cat No.: 631-0133). These were fixed in place by a drop of glue and dried.

Pertex™ (Cell Path, UK), a solvent based glue, required counterstained tissue to be de-hydrated and cleared in xylene. Dehydration was almost the reverse of the rehydration protocol followed in section 2.6.1.3. Slides were rinsed in water then immersed in 70% alcohol for 20s, 85% alcohol for 20s, 95% alcohol for 20s, Absolute Alcohol (AA) for 20s x2, HistoClear™ for 5mins and finally Xylene for 5mins.

Permafluor™ (Beckman Coulter, Cat No.: PNIM0752), a water based glue, required no further treatment of the tissue after excess counterstain had been rinsed off. More prone to air bubbles and incomplete rigidity after drying than Pertex, hence usually only used where dehydration was incompatible with reagents used, typically fast blue and fluorescent dyes.

2.6.1.12 Storage

All H&E, DAB and Fast Blue stained slides were stored in a dry, room temperature environment after review. They would be available for review indefinitely. Those slides labeled with fluorescent dyes, faded within 2 weeks of the experiment being run and were not stored.

2.6.2 Western Blotting

Western blotting separates protein mixtures according to molecular size by exploiting their electro-mobility through a gel with specific sized pores. These proteins are then transferred to a membrane that is then labeled with antibodies specific to the protein of interest. The antibody binding is then visualised and quantified. The details of this protocol are outlined below.

It is described as a Western Blot because it shares some similarities with the Southern Blot, invented by EM Southern, a British biologist.

2.6.2.1 Protein extraction

Experimental tissue, stored at –80^{O}C, was weighed whilst frozen where possible then defrosted on ice. Meanwhile, a lysis buffer "RIPA" was prepared and protease inhibitors freshly added. RIPA lysis buffer was used to break open the tissue and cells to release their proteins. The lysed tissue was then separated by centrifugation and the protein enriched supernatant stored at –80^{O}C until needed. The protease inhibitors prevented any protein degradation.

The RIPA buffer was made up from a 5x stock, diluted with distilled water and 10x protease inhibitor (Sigma, P8340). RIPA recipe: 15ml 5M NaCl, 25ml 1M Tris-HCl pH 7.4, 5ml 0.5M EDTA, 5g deoxycholate sodium, 0.5g SDS made up 100ml with distilled water. SDS was added to break non-covalent bonds within proteins and to give each protein a negative charge to facilitate their separation by PAGE.

Prepared RIPA was added and the mixture homogenised. Homogenisation helped disrupt the tissue integrity and was facilitated by a cordless handheld motor driven grinder (Sigma, Z35,997-1) with a pestle designed to fit the 1.5ml tube (Sigma, Z35,996-3). Approximately 100µl of lysis buffer was added per mg tissue. Following the homogenisation of the tissue into a suspension, it was incubated at 4°C for 1h. This incubation step improved the amount of protein recovered from the small pieces of tissue used (Laslett et al., 2000). The tissue lysate was centrifuged at 2500rpm for 10 minutes at +4^{O}C after which the protein rich supernatant was transferred to labeled, fresh 0.5ml eppendorf™ tubes and stored at -80^{O}C until used.

2.6.2.2 Protein Quantification

In order to make a relevant comparison between tissue samples, the total concentration of protein per extract needed to be quantified. A routine protein quantification method was established by Lowry in 1951, which has since been modified and commercialised by BioRad to produce a fast reliable method. This method relied on a standard curve of known protein concentration *vs* sample optical density at 450-750nm, usually 650nm. A scatter plot was used to generate the standard curve and a linear regression equation and the correlation coefficient, as calculated using Excel software (Microsoft, USA).

The standard protein used was Bovine Serum Albumin (BSA) as provided by BioRad at 2mg/ml and serially diluted in-house in prepared RIPA to maximise standardisation between standards and unknowns. At least five standards were used per quantification run. The optical density of the protein concentration was proportional to the colour change. An example of a standard curve and regression equation generated is shown in Figure 2.6. Occasionally, such a low concentration of protein was recovered in the lysis buffer that it was almost undetectable using the quantification method described. These samples were concentrated by removing the liquid buffer by freeze-drying at –80°C (Spin Freezer Model, Life Sciences International, UK) and re-solubilising the frozen powder in a smaller volume of buffer.

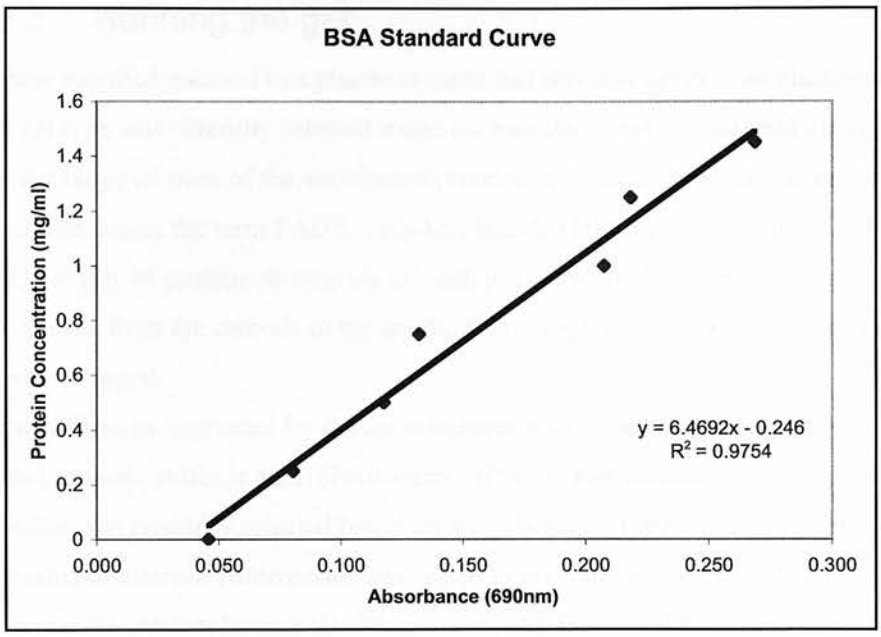

Figure 2.6: A typical calibration curve for protein concentrations, quantified against optical density of BSA standards at 690nm. Based on triplicate analysis of up to 7 standard solutions across the linear range. The values were processed by Microsoft Excel software and produced reproducible linear correlation coefficients (typically $R^2 > 0.95$). The curve was linear across the entire range set between 0.25-1.5mg total protein/ml.

2.6.2.3 Electrophoresis

The amount of protein used was restricted by the sample availability, concentration and the finite volume of the wells in the gels. All the products needed to perform the Western blot were supplied by Invitrogen unless specified otherwise.

2.6.2.3.1 Sample preparation

The protein samples (typically 10μg) were heated (70°C, 5mins) with 1:4 v/v LDS sample buffer, a glycerol rich dyed loading buffer (Invitrogen, NP0007) and 1:10 v/v reducing agent (Invitrogen, NP0009). This mixture was carefully loaded into the wells of a pre-cast 10% Bis-Tris gel (Invitrogen, NP0315BOX). These gels contained two parts, a large pored stacking gel that contained the wells and concentrated the sample prior to separation and a separating gel with smaller pores.

A pre-stained multi-coloured standard for SDS-PAGE (Invitrogen, LC5725) was loaded in at least one lane per gel to enable monitoring of the protein migration during electrophoresis. The marker included proteins of known molecular weight that could be used to compare against the expected position of the target protein.

2.6.2.3.2 Running the gel

Each gel was supplied encased in a plastic cassette and this was setup in an electrophoresis tank. The gel type was carefully selected based on manufacturers recommendations regarding the range of sizes of the anticipated proteins of interest. The gel was made from polyacrylamide, hence the term PAGE: PolyAcrylamide Gel Electrophoresis. SDS-PAGE relies on the ability of proteins to migrate through polyacrylamide gel pores when under an electrical charge, from the cathode to the anode, following treatment with SDS to make them all negatively charged.

The tank was filled as instructed by the manufacturer with a running buffer (MOPS: 3-(N-morpholino) propane sulfonic acid) (Invitrogen, NP0001) and attached to a power pack. The running buffer was carefully selected based on manufacturers instructions and approximate size of proteins of interest. Antioxidant was added to maintain the denatured form of the proteins during the electrophoresis (Invitrogen, NP005). Gels used had 10-15 lanes and ran at 200v for 50 mins.

2.6.2.4 Western transfer of proteins

Having separated the proteins according to their molecular weight (kDa), they were transferred from the gel and immobilised onto pre-cut PVDF membrane (Invitrogen, LC2002). The dry membrane was rehydrated by immersion in methanol for 30s, dH_2O for 2mins then transfer buffer for at least 15mins. A gel/ membrane sandwich was assembled in accordance with the manufacturers recommendations, and secured in a transfer tank. The sandwich arrangement of the gel and the membrane meant that the SDS treated proteins moved laterally from the gel towards the anode until their migration was halted by the membrane. The tank was filled as instructed by the manufacturer with a methanol rich transfer buffer (in-house protocol, see below) and re-attached to the power pack. The transfer took 4h at 40v or overnight at 20v.

Transfer buffer: per litre: 200ml Methanol, 14.4g Glycine, 3.03g Tris, made up with dH_2O.

2.6.2.5 Staining of gels

Following Western transfer of the separated proteins from the SDS gel to the PVDF membrane, the gel was stained to highlight residual proteins and check that the electrophoresis and blotting processes had worked. The gel was stained for 1h at room temperature using 20mls Gelcode Blue™ stain reagent (PerBio, Prod.24592) then rinsed in dH_2O overnight. This staining check was carried out routinely to ensure that each gel had run and transferred properly, without damaging the blot. Reviewed gels were discarded.

2.6.2.6 Protein Detection

Antibodies were used to identify proteins supported on the membrane, to facilitate their detection.

2.6.2.6.1 Blocking of non-specific binding

As for the immunohistochemistry performed on sections of fixed tissue (section 2.6.1), an initial block was used to saturate non-specific binding. At least 10mls of TBS-Tween buffer with 2% BSA (w/v) (Sigma) was poured over each blot which was then left to incubate for at least 1h at room temperature, on a rocker.

2.6.2.6.2 Primary antibodies

Following incubation, the blot was rinsed in TBS-T for 2 x 5mins then replaced with the primary antibody, directed against the protein of interest. The same primary antibodies were used as for immunohistochemistry (section 2.6.1.5) but 10x less concentrated, made up in TBS-T.

TBS-Tween® buffer: 0.05M Tris buffered saline at pH7.4 (TBS) + 0.05% (v/v) Tween® (Sigma). Blots were rocked (20-40rpm) for either 2 x 5 mins or 3 x 10mins with fresh buffer.

2.6.2.6.3 Secondary antibodies

The secondary antibodies used on the Western blots were HRP-conjugated and directed against the primary antibody host species as for immunohistochemistry (section 2.6.1.5). The HRP reacted with the chemiluminescent substrate to create light emission at the site of antigen expression. The specific pattern of light emission was captured by timed exposure to photosensitive film in a dark room. The Enhanced Chemi-Luminescence reagents (ECL Plus™, Amersham Life Science) were prepared immediately prior to use, after equilibration to room temperature, in accordance with the manufacturers advice. Exposure duration was adjusted according to the level of intensity achieved. The film used was designed especially for use with ECL reagents (Hyperfilm™, Amersham Life Science). Exposed film was developed by hand using Kodak reagents.

Expression levels of different proteins visualised in the same sample were quantified using Image Quant computer software as detailed in section 2.6.3.1.

2.6.2.7 Stripping of blots

Bound antibodies could be stripped away and the same blot re-probed for another protein. This enabled the same blot to have different protein expressions quantified against each other. This was particularly useful when experiments involved only small amounts of tissue. Blots could be stripped and re-probed successfully up to 4 times, though care was taken to

avoid using more than one primary antibody that had been raised in the same species to minimise background staining.

Antibodies were stripped by washing the blot after ECL exposure in TBS-T for 2 x 5mins then with 10-20mls of Restore™ (Pierce, Prod.No.:21059) at room temperature for 15-60 mins. Some monoclonal antibodies required a more severe method of stripping. This involved incubation of the blot in a rotating hybridisation chamber at 70°C for up to 1h with the in-house buffer detailed below. This was carried out in a fume hood

Stripping buffer: 1.875ml 62.5mM Tris pH 6.8, 211µl 100mM 2-β-Mercaptoethanol and 2% SDS (w/v) per blot.

2.6.3 Image Analysis

The level of protein expression could be analysed using computer assistance from immunostained fixed tissue with Image-Pro Plus software and from developed film after exposure of Western blots with ImageQuant software. More details are provided below.

2.6.3.1 Image-Pro® Plus

Image-Pro® Plus version 4.5 (Media Cybernetics, Inc., USA) computer software was used to accurately measure the distribution of positive staining of immunostained tissue sections. Various different approaches were used but they all required digitisation of the tissue image, collected using a microscope with a motorised stage (Prior Scientific Instruments Ltd., Cambridge, UK). and camera. Further analysis was undertaken using Sterology-pro-5.0 software (Datacell, Media Cybernetics, Inc., USA).

2.6.3.1.1 Determination of Sertoli cell nuclear volume/number per testis

Standard stereological methods were used to determine whether Sertoli cell nuclear volumes and/or number per testis were altered after treatment. Cross-sections of testes, previously immunostained for the Sertoli cell nuclear protein WT-1, were examined by light microscopy under oil-immersion. Three representative sections from each testis were selected and immunostained for WT-1. The three sections chosen were those corresponding to approximately 25, 50, and 75% intervals through the serially sectioned testis; at e21.5 this corresponded to sections that were 20–30 sections apart from each other. Image-Pro Plus 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) was used to analyse images captured using an Olympus BH-2 microscope fitted with a Prior automatic stage. An area of interest (AOI) was created by drawing around the perimeter of the cross-section (at x40 magnification). The software was used to select random fields within the AOI for counting and to place a grid over the tissue. The number of

fields counted per animal (30 per slide) was dependent on obtaining a percentage standard error value of <5%. The manual tag tool was used on Image-Pro® Plus to label all points falling over the nuclei of Sertoli cell nuclei. These were scored and expressed as a percentage of the total points counted, equivalent to the % of volume occupied by the cell type counted (i.e. Sertoli cell % volume occupancy).

For each animal, the values for percentage nuclear volume were converted to absolute nuclear volumes per testis by reference to testis volume (=weight) as shrinkage was minimal. Sertoli cell nuclear volumes per testis equate to cell numbers per testis, assuming no change in average nuclear size. However, as the individual nuclear volume of Sertoli cells might be influenced by treatment, average Sertoli cell nuclear volume was also determined.

Absolute volume (mg) = (% volume occupancy /100) x testis weight (mg)

Briefly, images were captured and analysed using the equipment described above. Nuclear measurements were made in Sertoli cell nuclei counterstained with haematoxylin as immunostaining masked the nuclear perimeters. An area of interest was created by drawing around the nucleus (at x100 magnification). Three diameter measurements (μm), which passed through the centre of the nucleus, per nucleus, were made using the Selector tool. This was measured for a minimum of 70 nuclei per testis, and the mean nuclear volume was then determined. Data for nuclear volume per testis was then converted to absolute numbers of Sertoli cell per testis by dividing by the average nuclear volume. The area per nucleus (μm^2) and the mean nuclear volume (μm^3) were calculated per sample by the software. The calculations assumed that the nuclei were spherical in shape. However, fetal Sertoli cell nuclei have irregular shapes but this assumption was made consistently for each of the treatments. Other experiments in our laboratory have shown that this method for determining Sertoli cell number yields comparable results to those obtained using an "unbiased" method (optical dissector) in which shape of the nucleus is not a factor e.g. Atanassova *et al* (2005).

$$\text{Number of cells (millions)} = \frac{\text{Absolute volume (mg)}}{\text{mean nuclear volume } (\mu m^3)} \times 1000 \text{ (to account for mg to } \mu m^3)$$

2.6.3.1.2 Proliferation Index (PI)

The PI is the percentage of cells (e.g. Sertoli cells) that positively express a proliferation marker, in this case BrdU (section 2.2.3). The number of BrdU positive and BrdU negative Sertoli cells was counted in at least 15 seminiferous cords per section/ per animal. Counts were made by tagging a captured image (as described previously, at x63 magnification) and tracked per section on a second screen with a tiled image (3x3 frames) taken at x20. Images were captured and analysed using the equipment described above.

$$PI\ (\%) = \frac{\text{Number of positively stained Sertoli cell nuclei}}{\text{Number of stained + unstained Sertoli cell nuclei}} \times 100$$

2.6.3.1.3 Determination of Leydig cell aggregation

Modified stereological methods were used to quantify the area of a testis cross-section represented by Leydig cells, to determine whether the distribution of Leydig cells throughout the testis was altered after DBP treatment (Mahood et al., 2005). Cross-sections of testes, previously immunostained for the Leydig cell cytoplasmic protein 3β-HSD, were examined by light microscopy under oil-immersion. Three representative sections from each testis were selected and immunostained for 3β-HSD. The three sections chosen were those corresponding to approximately 25, 50, and 75% intervals through the serially sectioned testis; at e21.5 this corresponded to sections that were 20–30 sections apart from each other. Images were captured and analysed using the equipment described previously. An area of interest (AOI) was created by drawing around the perimeter of the cross-section (at x40 magnification).

Previously specimens immunostained for 3β-HSD are usually of sufficient homogeneity, high contrast, and low background to allow computer-assisted thresholding and subsequent computer-assisted counting of Leydig cell (3β-HSD -immunopositive) clusters and determination of Leydig cell cluster area (Mahood et al., 2005). In the present experiments, counterstained pyknotic nuclei in cultured testis explants were of equally high contrast so a modified method was adopted. Computer-assisted segmentation was used as it could distinguish between colours, i.e. brown stained Leydig cells and blue stained pyknotic nuclei, rather than converting the captured image to greyscale as for thresholding. Segmentation was used to identify and analyze clusters of 3β-HSD -immunopositive cells, generating data on cluster number, area, and the proportion of each section occupied by Leydig cell clusters.

2.6.3.2 Image Quant TL ®

Following Western analysis, developed Hyperfilm was scanned to generate a greyscale ".tif" file. The image was analysed by ImageQuant (Amersham Life Sciences, UK) and the area of film exposed to the chemiluminescence was quantified per gel, per antibody and per sample. The area of exposure equated to the amount of labeled protein present in the sample.

2.7 Hormone Analysis

Endocrine effects of *in vivo* and *in vitro* treatments were quantified by measuring the levels of testosterone (Leydig cell product) and inhibin B (Sertoli cell product) within either media retrieved at the end of *in vitro* experiments or in whole testis homogenates from *in vivo* experiments. Protocols were already established for these hormone assays, though some optimisation was required for these sample conditions.

It has been reported that pregnenolone contributes very little to the total steroid content at any one time, perhaps due to the omnipresence of 3β-*HSD*, but also making it a poor endpoint to use for assessment of any steroidogenic disruption, hence the use of testosterone (Payne and Youngblood, 1995).

2.7.1 Testosterone

This steroid hormone had two in-house assay protocols established: one involving a radioisotope labeled antigen and another a radiation free ELISA plate assay. Due to Health and Safety regulations, and waste disposal costs, the radiolabelled assay is being phased out. Both methods were used and are detailed below. For both methods, samples were assayed in duplicate after appropriate dilution. Aliquots of standards, controls and quality control media were incubated alongside samples for every assay run.

Unlike in the media samples, the whole tissue homogenates underwent an additional extraction step to free up any testosterone bound in the tissue to proteins such as albumin.

2.7.1.1 Extraction of steroids from whole organs

Whole fetal testes were homogenised in chilled 0.5ml 0.1M PBS buffer. 100µl aliquots were placed in glass test tubes and vortexed for 5 minutes with 2ml di-ethyl ether. The shaken tubes were placed in a bath of methanol and dry ice to freeze the aqueous portion, revealing the unfrozen organic portion that was decanted into clean tubes. These tubes were left over night in a fume hood to enable the organic solvents to evaporate. The dry residue was re-suspended in assay buffer and stored at –20°C until quantified. This method has an extraction efficiency of >85% in this laboratory.

2.7.1.2 Radioimmunoassay (RIA)

This fluid phase technique measured the level of competition between a fixed concentration of radiolabelled antigen (testosterone with I^{125}, APB Biotech, UK) against the unknown quantity of unlabelled antigen in the test sample. The more antigen in the test sample, the lower the level of binding by the labeled antigen. After a three-hour incubation at room temperature, a second antibody (Donkey-anti-sheep DAGS), raised against the sheep IgG, was added and tubes were left overnight at 4°C to bind with the testosterone/antibody complex to form a stable precipitate. The incubated tube received 2ml of wash buffer. The amount of radiation in the precipitate was measured and the test sample values were compared to a standard curve of known antigen concentrations. A gamma counter (Multigamma 1261, LKB Wallac, Turku, Finland) was used to measure residual I^{125}.
This is a precise and sensitive assay with a low intra-assay coefficient of variance (<10%). Method blanks, quality control (QC) samples (spiked buffer), and standards were analysed along with media samples. Where samples are presented on one graph, these were analysed simultaneously. If the QC values measured outside 2 standard deviations from the mean, the assay was rejected, this happened rarely. The source of the deviation (usually human error!) was determined and corrected before the analysis was re-run. The inter-assay (6 runs) coefficient of variance of the three QC samples ± standard errors are listed below. The mean coefficient of variance = 16.5%. The limit of detection for this assay was 8pg/100µl.

- Low: 7.9 ± 0.4pg/100µl = 13.0%
- Medium: 26.5 ± 1.81g/100µl = 16.8%
- High: 135.7 ± 19.7pg/100µl = 19.7%

Data were expressed as pg testosterone per 100µl sample. Results were analysed using a computer programme (AssayZap, BioSoft, Cambridge, UK) specifically designed for this purpose. For presentation purposes, data were converted to ng/ml media (= pg/100µl x 0.01).

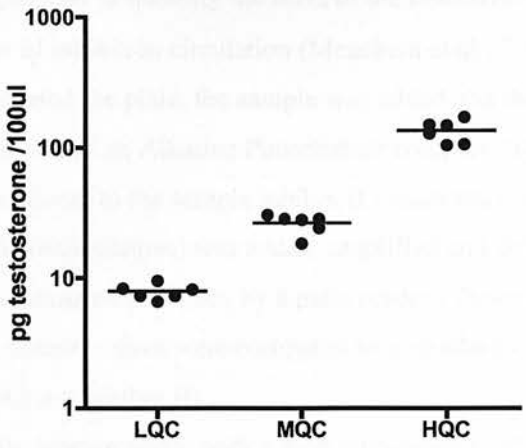

Figure 2.7: Quality control plot for testosterone. The scatter plot shows the actual values obtained and the median per QC. The graph is on a logarithmic scale to accommodate the concentration range of the control samples.

2.7.1.3 Enzyme linked ImmunoSorbent assay (ELISA)

2.7.1.3.1 Testosterone

This solid phase technique measured the level of competition between a fixed concentration of biotin-testosterone-testosterone (Sigma, T2168) and sample testosterone. A second antibody (Donkey-anti-sheep, Binding Site, UK), raised against the sheep IgG, bound with the testosterone/antibody complex to fix it to the plate. Unbound components were washed away. The residual amount of biotin-labelled testoterone bound to the plate, was detected using streptavidin-horseradish peroxidase (Amersham, UK) and resulted in a visual colour change inversely proportional to the sample testosterone concentration. The colourimetric change was quantified at 450nm by a plate reader (Victor). The colour change was measured and the test sample values were compared to a standard curve of known antigen concentrations. Data were expressed as pg testosterone per 100µl media collected. Results were analysed using a computer programme (AssayZap, BioSoft, Cambridge, UK) specifically designed for this purpose. For presentation purposes, data were converted to ng/ml media (= pg/100µl x 0.01).

2.7.1.3.2 Inhibin-B

Levels of this dimeric glycoprotein hormone were quantified in using an established in-house solid phase technique (Sharpe et al., 1999). This sandwich assay relies on two antibodies, raised against different parts of the inhibin B molecule: the *capture antibody* "C5" raised against the N-terminus of the β_B subunit and the F(ab) fraction of the *label antibody* "R1" was raised against the N-terminus of the inhibin α subunit. By using both of

these antibodies, it is possible to quantify the level of the bioactive inhibin dimer (α-β_B) from the various forms of inhibin in circulation (Meachem et al., 2001).

The capture antibody coated the plate, the sample was added and the labeled antibody added. The labeled antibody included an Alkaline Phosphatase complex. This caused a visual colour change inversely proportional to the sample inhibin B concentration when the ELISA substrate solution (Life Technologies) was added, amplified and developed. The optical density changes were quantified at 490nm by a plate reader (Victor). The colour change was measured and the test sample values were compared to a standard curve of known antigen concentrations (recombinant inhibin B).

This is not a particularly precise assay, with a high intra-assay coefficient of variance (>15%). Method blanks, quality control (QC) samples (spiked buffer), and standards were analysed along with unknown used media samples. Where samples are presented on one graph, these were analysed simultaneously. When QC values measured outside 2 standard deviations from the mean, the assay was rejected. The source of the deviation was determined and corrected before the analysis was re-run. The inter-assay (8 runs) coefficient of variance of the three QC samples ± standard errors are listed below (Figure 2.8). The mean coefficient of variance = 20.6%. The limit of detection for this assay was 99pg/100µl.

- Low: 99.5 ± 12.1 pg/ml = 32.1%
- Medium: 162.8 ± 9.4 pg/ml = 16.3%
- High: 293.4 ± 13.9 pg/ml = 13.4%

Data were expressed as pg inhibin-B per ml media sampled. Results were analysed using a computer programme (AssayZap, BioSoft, Cambridge, UK) specifically designed for this purpose. For presentation purposes, data were converted to ng/ml media (= (pg/ml) /1000).

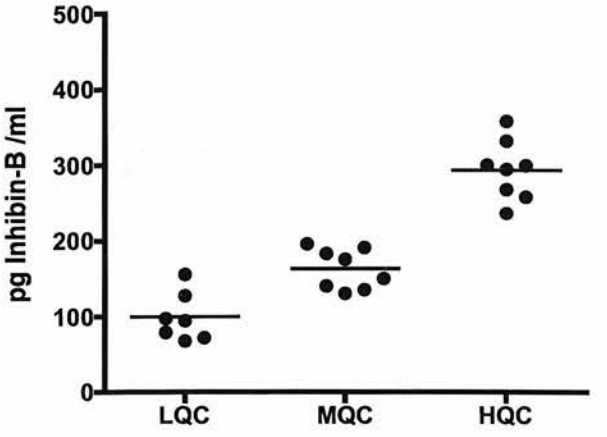

Figure 2.8: Quality control plot for inhibin-B. The scatter plot shows the actual values obtained and the median per QC.

2.8 Statistical analysis

Statistical analysis was used to evaluate morphological parameters and hormone concentrations.

The difference between two means was determined using the unpaired two-tail t-test, with or without logarithmic transformation of the data to obtain normal distribution.

The difference between more than two means was determined using analysis of variance (one-way ANOVA). Where more than 4 values were present in a column of data, Bartlett's test for equal variances was applied and if significant, data were subjected to logarithmic transformation of the data to obtain normal distribution. The R^2 value of a graph, where reported, reflects the variability of the group means within the variability of the groups, meaning a high value (close to 1) indicates that a large fraction of the variation seen is due to the treatments applied to the groups.

Various post ANOVA analyses were undertaken where appropriate. The test for linear trend assessed whether there was a trend across columns of data arranged in a systematic sequence. Dunnett's test compared data sets from treated groups against the control data set within an experiment, but not against each other. Boneferroni's test was used to compare all possible combinations of pairs of columns within an experiment except where >5 columns of data were compared, when the Tukey-Kramer test was applied.

Where appropriate, values were presented as means ± S.E.M. The criterion for significance for all tests was set at $P< 0.05$. Specific software was used to assist in the data analysis (GraphPad Prism v4.0b for Macintosh, GraphPad Software, San Diego, USA).

3 Studies using long-term exposure of the fetal rat to DBP

In 2001, Skakkebaek *et al* described a hypothesis that the four male reproductive disorders, collectively described as Testicular Dysgenesis Syndrome (TDS) all stemmed from the disruption of gonadal development during fetal life, (Skakkebaek et al., 2001). It was theorised that the recent and rapid rise in the incidence of TDS, across few generations and many countries, was contributed to by the influence of environmental factors, which may affect change upon genetically predisposed individuals. They went on to propose that as these disorders all originated during fetal life, exposure to factors such as endocrine disruptors during this vulnerable window of development must be considered as a potential contributor to the rise in incidence of TDS. This attractive model might well account for many of the reported epidemiological findings. The paper went on to emphasise that further research was needed to explore the risk to humans posed by chemicals with the potential to inflict disruption on normal development and whether any protection for the reproductive health of future generations could be outlined.

3.1 Introduction

In order to understand more about this syndrome and investigate the proposed common fetal source, it became imperative to develop an appropriate animal model. Given that the TDS hypothesis suggested that exposure to chemicals capable of endocrine disruption could be a risk factor, it seemed plausible that a known chemical could induce a profile of symptoms in animals under laboratory conditions akin to TDS in man.

Various chemicals are known to affect male fertility. The profile of their effects on male reproduction is specific to the mechanism of toxicity induced by each compound. Chemicals can be categorised according to the profile of effects that they cause (Gray et al., 1999). By reviewing the toxicity profiles of known testicular toxicants, it was possible that a candidate to induce TDS would become apparent.

3.1.1 Phthalates

One class of compounds known to affect the male reproductive tract is the phthalates. These anthropogenic chemicals are widely used, such as in the plastics industry to broaden the function of the rigid plastic poly vinyl chloride (PVC). Since the 1970's, phthalates have been investigated for their potential toxicity due to their high level of use (Sharpe, 2001). There are many different phthalates with slightly different chemical properties hence their varied industrial uses and potential toxicity. For example, di-ethylhexyl phthalate (DEHP) is

a well-known hepatic peroxisome proliferator (peroxisomes are hepatic organelles that metabolise xenobiotics) and a Sertoli cell toxicant. These properties were revealed through toxicological assessment using laboratory animals (Dalgaard et al., 2000; David et al., 2000a; David et al., 2000b; Mann et al., 1985; Oishi, 1986).

The majority of toxicology studies were carried out in young/ adult rodents with DEHP administered *via* the food, as DEHP absorbed through the gut and dietary administration represents the most widespread exposure risk for humans (David et al., 2000a; Kanda et al., 1990)(Kanda et al., 1990). All organs were examined for histopathology after 104 weeks of exposure to DEHP at up to 6000ppm (mice) or 12500ppm (rats). Liver tumours were observed at DEHP exposure levels >500ppm and peroxisome proliferation was evident in mice exposed to 100ppm but not in rats below 500ppm. Bilateral aspermatogenesis was observed in the testes of rats exposed to >500ppm and hypospermia in the 6000ppm exposed mice (David et al., 2000b). Uterine weights in the 6000ppm exposed female mice group were significantly lower than for the controls (David et al., 2000a). These studies confirmed that chronic DEHP exposure did adversely effect the liver and testis pathology of rodents exposed to >500ppm, but did not reproduce the syndrome of disorders comprising TDS. The exposure timeframe and/or the chemical would have to be reconsidered.

Reports of chronic exposure of rodents to different phthalates were reviewed. For example, Di-(C_7-C_9 alkyl) phthalate (D79P) is used as a plasticiser for PVC in flooring and fabric-coating and Di-(C_9-C_{11} alkyl) phthalate (D911P) is used as a plasticiser for PVC in roofing and automotive applications. Continuous chronic exposure of rats to 2.0% D79P or D911P via the diet, across two generations, did not impair reproductive function of the F_0 or the F_1 offspring, even at levels that induced systemic toxicity and peroxisome proliferation typical of this class of chemicals (Willoughby et al., 2000). This was not consistent with the TDS phenotype either.

Further studies with DEHP changed the timeframe of exposure and the dosing route. Sprague-Dawley rat dams were dosed by gavage with corn oil or with up to 1,500 mg DEHP/kg/day from e3 through to postnatal day (d) 21. Dose-related effects on the F_1 male offspring included a high incidence of anterior prostate agenesis, a lower incidence of partial or complete ventral prostate agenesis, occasional dorsolateral prostate and seminal vesicle agenesis, reduced sperm counts, and testicular, epididymal, and penile malformations (Moore et al., 2001). This profile of effects was more consistent with the TDS hypothesis, including the exposure during perinatal development. However, the prevalence of anterior prostate agenesis following *in utero* and lactational DEHP exposure appears to be unique

among all chemicals but is inconsistent with the malformations of the male reproductive tract associated with the TDS phenotype (Moore et al., 2001).

The alterations in the development of the reproductive tract of male rats exposed to DEHP *in utero* and during suckling were deduced to be associated with disruption of fetal testis testosterone production (Parks et al., 2000). This was consistent with the proposed TDS hypothesis of disrupted gonadal development during fetal life (Skakkebaek et al., 2001). The DEHP studies reported by Parks *et al* (2000) were followed up with a comparison of perinatal exposure of male rats between six different phthalate esters. Of these, only three adversely affected sexual development (DEHP, BBP and DINP) but three (DOTP, DEP and DMP) did not induce any changes at 750mg/kg/day from e14.5 to postnatal day 3 (Gray et al., 2000). These data confirmed that the chemical structure of the phthalates affected their potency and only those monoesters with an ester group 4-6 carbons long are developmental toxicants (Gray et al., 2000).

3.1.2 Di-*n*-butyl phthalate

One such phthalate is Di-*n*-butyl phthalate (DBP, $-CH_2(CH_2)_2CH_3$). DBP was found to be the most common phthalate ester with reprotoxic potential, that was measured in the urine of American women of child bearing age (Blount et al., 2000). DBP is used as a plasticiser in PVC products as well as a solvent in some personal care products (Foster et al., 2001). DBP causes a variety of alterations of normal fetal development, depending on the dose and window of exposure. For instance, pregnant rats given DBP by gavage at either 750, 1000 or 1500 mg/kg/day during early gestation (up to e15) (Ema et al., 1994) presented fetuses with a range of terata:

- 100% post-implantation loss in the 1500 mg/kg/day dose group
- increased vertebral column malformations with 750 or 1000mg DBP/kg/day on e7-9
- dose-dependent increased external and internal malformations such as cleft palate/ fusion of the sternebrae with 750 or 1000mg DBP/kg/day on e13-15

Exposure to DBP (400-650 mg/kg/day) in rodent and non-rodent species, during late gestation, has resulted in a variety of organogenesis related malformations that persisted into adulthood, just like TDS. In the parental generation, effects on reproduction were modest (small decreases in litter size and pup weight following treatment) as seen with chronic DEHP exposure. However, the F_1 male offspring had marked decreases in fertility (at 650 mg/kg/day), with reduced sperm counts and reproductive tract malformations observed in adulthood (Foster et al., 2000).

Multiple studies have investigated the effect of high levels of DBP exposure *in utero* on the development of the male reproductive tract (Foster et al., 2000; Foster et al., 2001; Marsman,

1995; Mylchreest et al., 1998; Mylchreest and Foster, 2000; Mylchreest et al., 1999; Mylchreest et al., 2002; Mylchreest et al., 2000). The gross changes in the pathology of the male reproductive tract induced by this treatment regime included:

- testicular atrophy
- decline in number of ejaculated sperm / increased number of abnormal sperm
- cryptorchidism
- hypospadias
- aplastic or absent epididymides
- reduced anogenital distance

This range of specific male and reproductive tract abnormalities seen in rodents following high-level DBP exposure during gonadal development, shares many parallels with human TDS. This encouraged further exploration of this compound to generate a model system to investigate the mechanisms that underlie TDS.

3.1.3 Experimental aims

However, at the time these studies were conceived, the specific histopathological changes induced in the rat testis by *in utero* DBP exposure compared with those associated with human testicular dysgenesis had not been reported. The aim of the present studies was to investigate the effect on the testis of *in utero* exposure to 500mgDBP/kg/day over e13-20 in the fetal rat at e21.5 and 500mgDBP/kg/day over e13-21 in the adult male rat and compare any findings with the reported TDS phenotype.

3.2 Methods and Materials

Further details on the general methods and materials used are listed in Chapter 2. Overall, time-mated rats were dosed with 500mgDBP/kg/day at 1ml/kg from e13.5 until e20.5 followed by necropsy on e21.5 or until e21.5 followed by necropsy of the adult F_1 males. Following collection of the tissue, samples were assayed for testosterone and inhibin-B levels or analysed for histological changes, with comparisons made between DBP exposed samples and the relevant vehicle exposed controls.

3.2.1 Statistical analysis

The significance of any difference between two values was determined by an un-paired two-tailed t-test, with or without logarithmic transformation of the data to obtain a normal distribution. Where Bartlett's test showed significant differences in the variances between the two data sets, even after log transformation, data were re-analysed using Welch's correction.

The criterion for significance for all tests was set at $P<0.05$ and n = number of testes examined, taken from at least 3 litters, unless otherwise stated.

3.3 Results

The aim of these studies was to investigate the effects of *in utero* DBP exposure on the developing testis of the rat. Fetal rats were exposed *in utero* to 500mgDBP/kg maternal bodyweight from e13.5 until the day prior to necropsy (e20.5) or the end of gestation (e21.5). Testes were collected for study on e21.5 or >d90 (adult) and compared with those recovered from age matched animals, dosed to the same regime but with the vehicle (Controls). Typically, the left testes were weighed, Bouin's fixed, paraffin embedded, sectioned and immunostained as required: the right testes were snap frozen until processed for protein or steroid level investigations. At e21.5, each major testis cell type was observed and analysed separately: Sertoli cells, gonocytes and Leydig cells. The peritubular myoid cells, that surround the developing testis cords, showed no obvious treatment related changes at low magnification reviews, so were not more closely investigated. The consequences of the changes observed in the fetal testes were followed up in adulthood.

3.3.1 Effect of fetal exposure to DBP on e21.5 testes

Testis weight was recorded in e21.5 rats and the mean testis weight per litter per treatment regime was calculated. DBP exposure induced a significant decrease in testis weight compared to control animals (Figure 3.1). The reason for this decrease in weight was investigated at a cellular level.

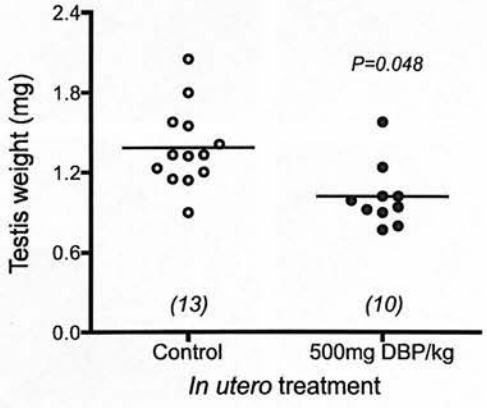

Figure 3.1: Mean testis weight per litter (mg) of e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Individual mean per litter (*n*) and mean per treatment (horizontal line) are plotted (*n* values are shown in parentheses). The DBP treated group showed a significant (P=0.048) decrease in testis weight compared to the control testes.

3.3.1.1 Sertoli cells

It has been shown that the number of Sertoli cells determines the capacity of the testis to produce sperm (Orth, 1982; Orth, 1984). The effect of DBP exposure on the number of

Sertoli cells per testis was quantified using established stereological techniques, detailed in Chapter 2. Prior to analysis, testis sections were immunostained for WT-1, a protein found in Sertoli cell nuclei (Figure 3.2). Low magnification review of the stained sections showed WT-1 cells at the periphery of the testis cords for both treatment groups. Higher magnification review of the sections showed normal WT-1 negative areas between the developing cords in control and treated animals whereas the testes from DBP-exposed animals showed areas of interstitial cells, some of which were WT-1 positive (Figure 3.2). This abnormal staining pattern, suggestive of interstitial Sertoli cells, could not be explained as a plane of section or non-specific staining artefact. This area was described as dysgenetic and its relevance was followed up in adult animals (Figure 3.26). Cells that were WT-1 immunopositive within the dysgenetic areas or outside a cord were discounted as these cells were unlikely to be capable of supporting germ cells so could not contribute to fertility in adulthood (Figure 3.2).

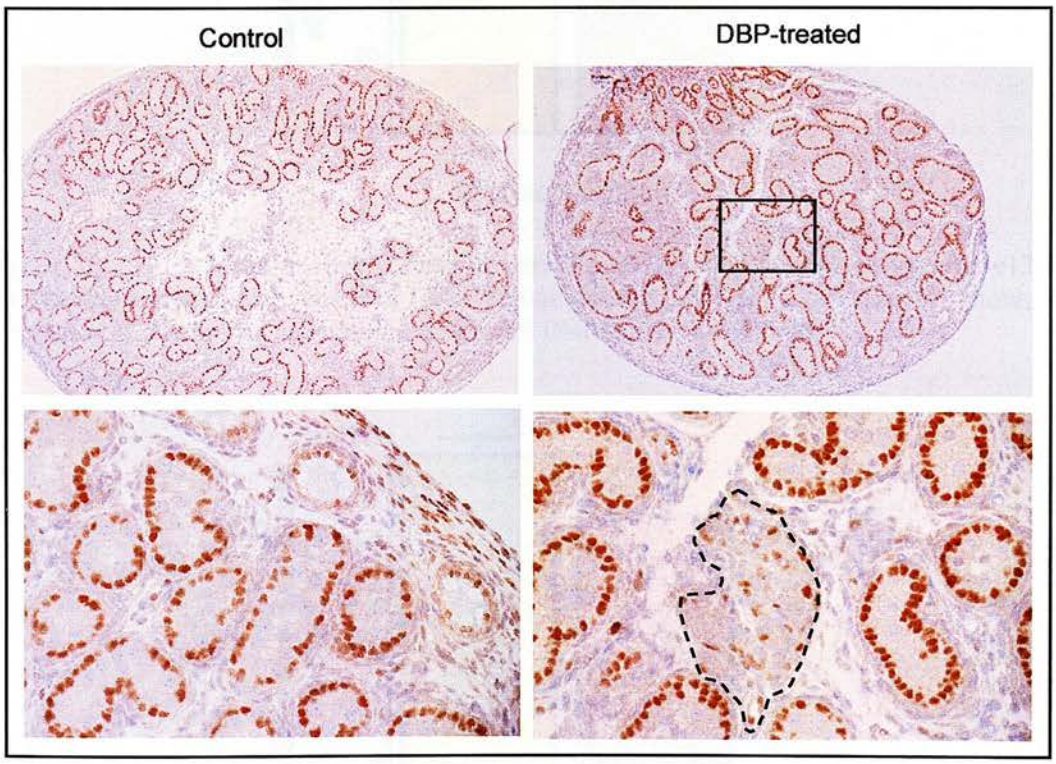

Figure 3.2: WT-1 immunostained sections of e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Note the positioning of stained Sertoli cell nuclei, at the periphery of the developing cords. Additional staining was seen within abnormal "dysgenetic" interstitial areas of the DBP exposed testis (dashed outline). The top panels were photographed using a x10 objective and a smaller area (solid rectangle) using a x40 objective.

Stereological analysis showed no significant effect of DBP on the mean nuclear volume of the Sertoli cells (Figure 3.3) but a slight decrease in the percentage of the testis occupied by Sertoli cells from a mean of 16.6% to 13.9% (Figure 3.4). These results suggested that though Sertoli cell nuclei was unaffected by DBP exposure, treatment may have slightly reduced their number. However, when these data were used to calculate the number of Sertoli cells per testis, there was no significant difference (P=0.07) between the treatment groups seen, though the data suggested a trend towards a decrease in the number of Sertoli cells per testis following DBP exposure (Figure 3.5).

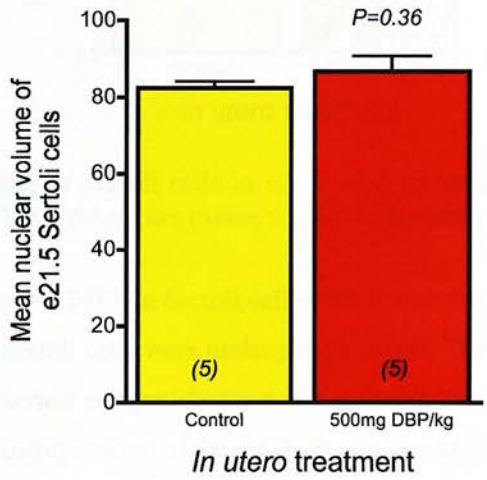

Figure 3.3: Mean nuclear volume of Sertoli cells in e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). There was no significant effect of DBP treatment (P=0.36).

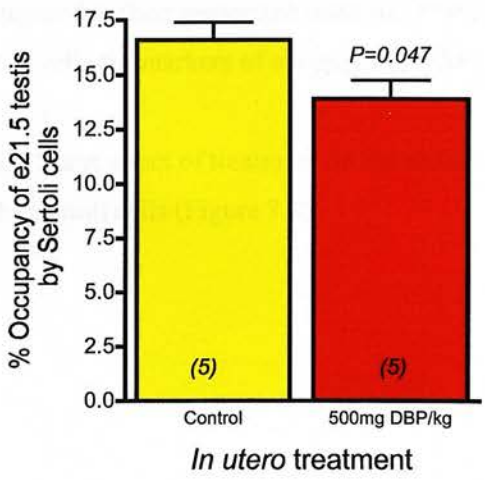

Figure 3.4: Percentage volume occupancy of Sertoli cells in e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). The DBP treated group showed a significant (P=0.047) decrease in the percentage occupancy of the testis by Sertoli cells, compared to the control testes.

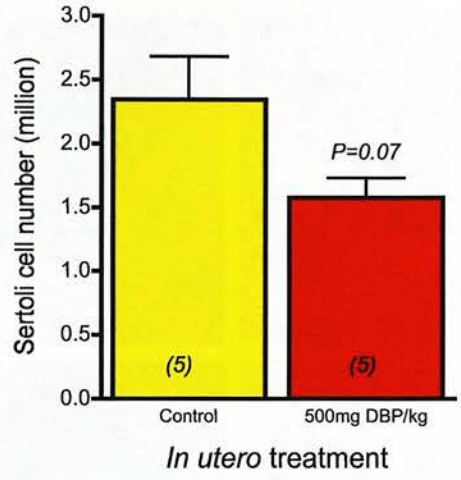

Figure 3.5: Number of Sertoli cells in e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses).

The non-significant effect of DBP on Sertoli cell number was followed up by investigation of the rate at which the Sertoli cells were undergoing mitosis. The effect of *in utero* DBP exposure on the rate of Sertoli cell proliferation was measured by immunostaining Bouins fixed testis sections for incorporation of the BrdU, injected into the mother 1.5h prior to kill. The Sertoli cell proliferation index was calculated from three pups per litter (*n*) (Figure 3.7). Proliferation was also evident in non-Sertoli cells (e.g. interstitial cells, Figure 3.6) and dysgenetic areas but this was not measured. No BrdU uptake was seen in any gonocytes. There was no significant difference in the proliferation index for Sertoli cells in the DBP exposed testes when compared to their respective controls (P=0.30) (Figure 3.7). Immunostaining of Sertoli cells for markers of apoptosis (TUNEL) showed no staining (not shown).

Finally, there was no significant effect of treatment on the testicular level of inhibin-B, a hormone produced by the Sertoli cells (Figure 3.8).

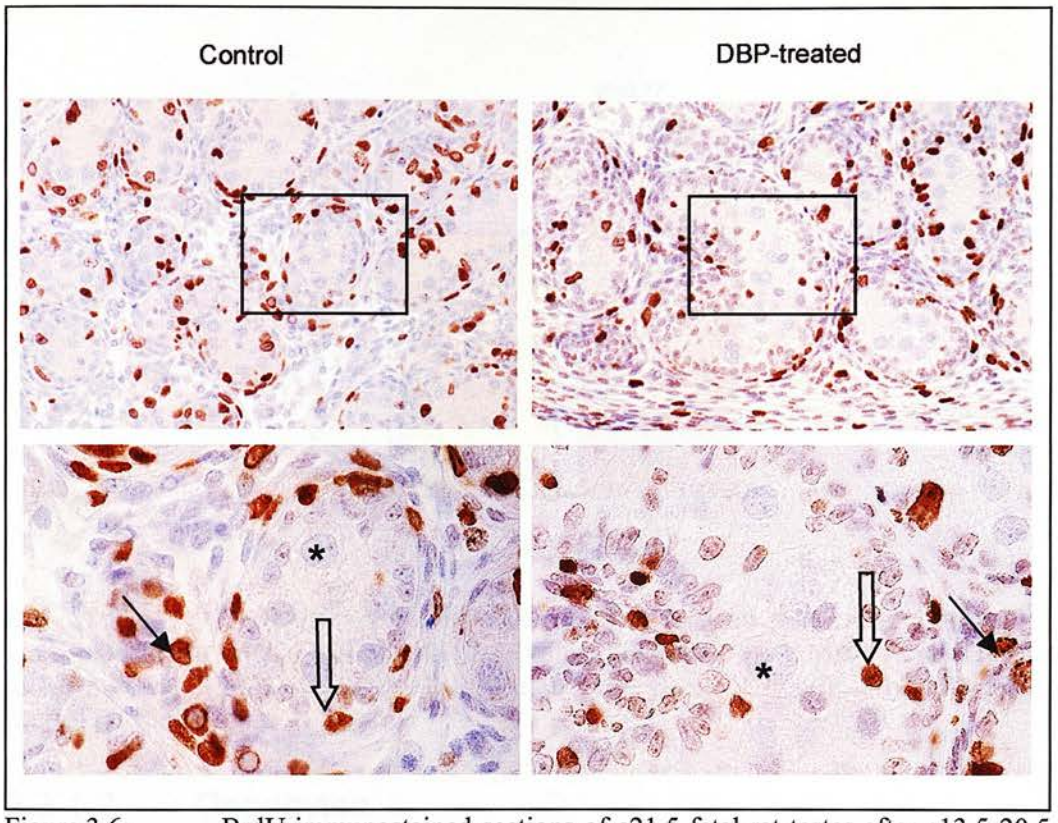

Figure 3.6: BrdU immunostained sections of e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. BrdU was administered 1.5h prior to testis collection. It was incorporated by the intratubular Sertoli cells (wide arrows). Interstitial cells were also proliferating at this age (narrow arrows) but gonocytes appeared quiescent (asterix). The top panels were photographed using a x40 objective and a smaller area (solid rectangle) using a x100 objective.

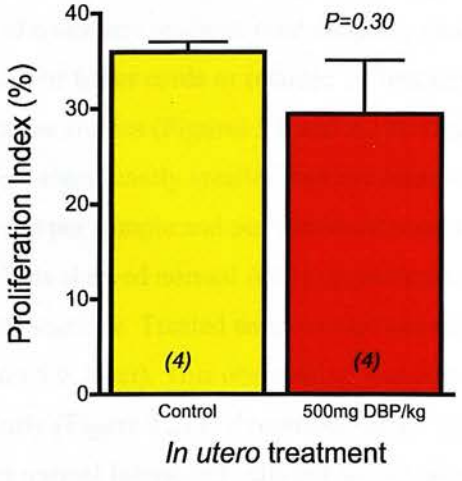

Figure 3.7: Proliferation Index (%) of Sertoli cells in e21.5 fetal rat testes after e13.5-20.5 *in utero* exposure to vehicle or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). There was no significant effect of DBP treatment.

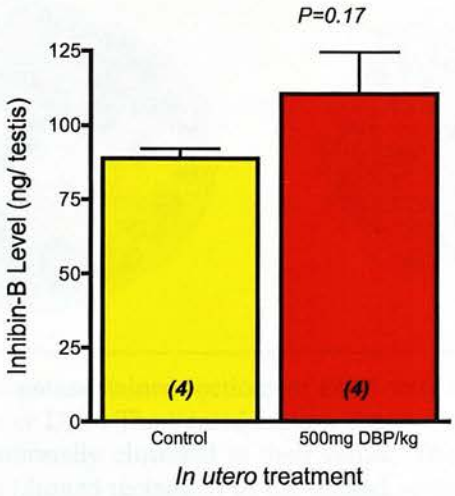

Figure 3.8: Testicular inhibin-B levels in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). The inhibin-B levels in treated testes were not significantly different from control testes (P=0.17).

3.3.1.2 Gonocytes

Testis sections were immunostained for AMH, a protein expressed in the Sertoli cell cytoplasm that surrounds the fetal gonocytes. Low magnification reviews of the AMH staining demonstrated that the number of cords present per testis after DBP exposure was reduced (Figure 3.9). This difference was quantified and revealed a significant reduction in the number of cords per section following DBP exposure, compared to the control sections (Figure 3.10). The effect of treatment on mean cord diameter was not quantified. Whether this reduction was the result of fewer cords or reduced coiling of the same number of cords could not be deduced by these studies (Figures 3.9 and 3.10). It should be noted that the testes exposed to DBP were significantly smaller than the control testes (Figure 3.1) and that the plane of section analysed per sample and per treatment were comparable.

Further review of the sections showed normal AMH negative areas between the developing cords in control and treated sections. Treated sections also showed areas between cords with AMH positive cells (Figure 3.9, inset). This observation was consistent with the WT-1 positive cells seen previously (Figure 3.2) in dysgenetic areas, supporting the suggestion that some of these cells are not normal interstitial cells but abnormally situated Sertoli cells.

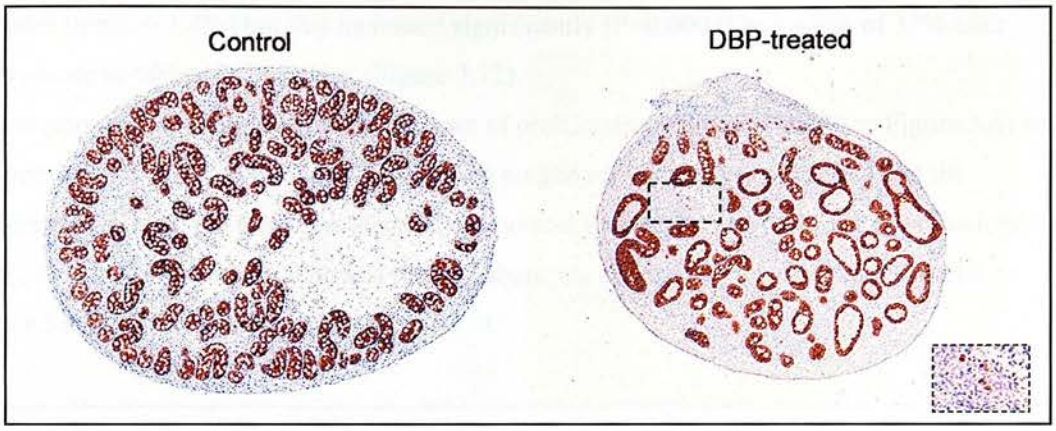

Figure 3.9: AMH immunostained sections of e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle or DBP. The treated section shows a reduced number of distended cords with gonocytes abnormally clustered in their centre. The inset shows AMH positive cells in a dysgenetic area (dashed rectangle) of the treated section. Photographed using a x4 objective.

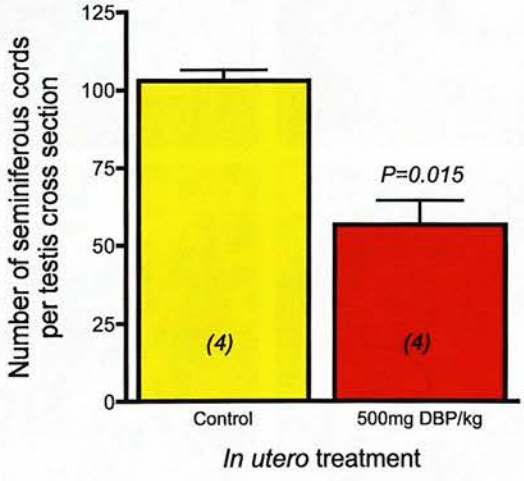

Figure 3.10: Number of seminiferous cords per cross section of e21.5 fetal rat testes ± *in utero* exposure to DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). The DBP treated group showed a significant (P=0.015) decrease in the number of seminiferous cords per testis cross section compared to the control testes.

Examination of AMH stained testis sections at higher magnification revealed the presence of abnormally nucleated gonocytes in testes from DBP exposed tissue (Figure 3.11). Normal e21.5 testes contain mononucleated gonocytes, with rare and infrequent multinucleated gonocytes (Boulogne et al., 1999). The effect of DBP exposure on the frequency of multinucleated gonocytes was quantified using established stereological techniques, detailed in Chapter 2. The percentage of cords per testis that contained at least one abnormal multinucleated gonocyte (MNG) was calculated. A few MNG's were seen in the control

testes (mean = 1.4%) but this increased significantly (P=0.0004) to a mean of 37% after exposure to 500mgDBP/kg/day (Figure 3.12).

Immunostaining of gonocytes for markers of proliferation (BrdU uptake, see Figure 3.6) and apoptosis (TUNEL, see Figure 2.1) showed no gonocyte staining, indicating that the abnormal gonocytes were quiescent, as per normal for this developmental stage (Boulogne et al., 1999). Further studies showed that the abnormal gonocytes appeared between e18.5-e19.5 but had disappeared by postnatal d10.

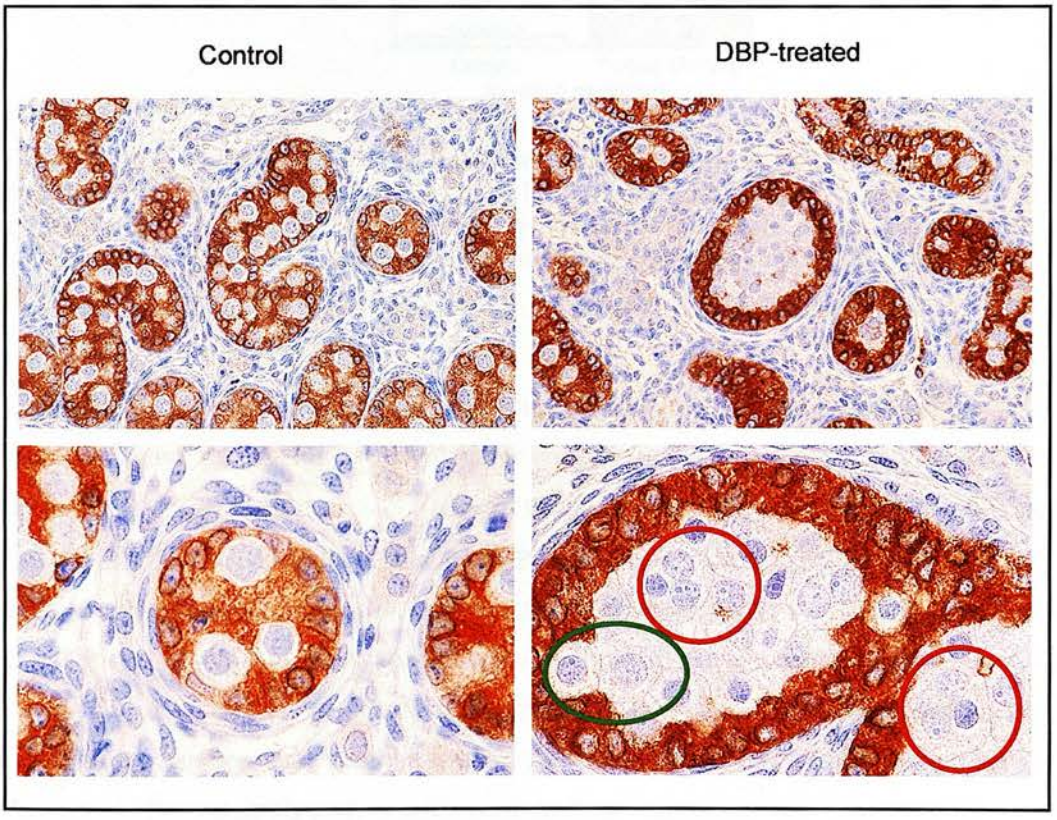

Figure 3.11 AMH immunostained sections of e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle or DBP. The Control sections show normal mononucleated gonocytes dispersed evenly across the cords (top left panel) compared to the distended cords in the DBP-exposed testis (top right panel). Examples of typical multinucleated gonocytes are highlighted in the bottom DBP-exposed testis (red circles) and remaining mononucleated gonocytes (green circle). The top panels were photographed using a x40 objective and the bottom panels were photographed using a x100 objective.

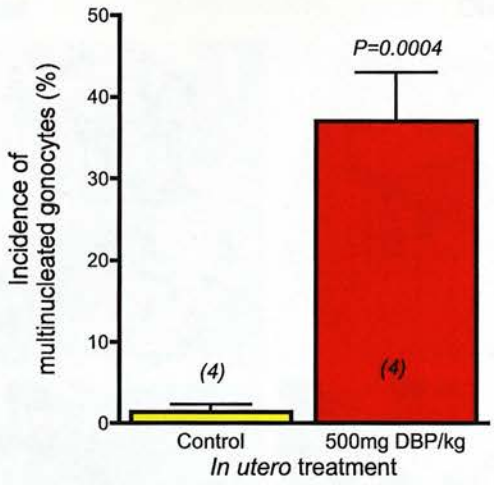

Figure 3.12: Incidence (%) of multinucleated gonocytes in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). The DBP treated group showed a highly significant (P=0.0004) increase in the incidence of multinucleated gonocytes compared to the control testes.

3.3.1.3 Leydig cells

Clusters of Leydig cells between the developing testicular cords were defined using immunostaining for 3β-HSD, a steroidogenic enzyme present in Leydig cell cytoplasm (Figure 3.13). Low magnification reviews of the 3β-HSD stained testis sections highlighted that the distribution of Leydig cells after DBP exposure was altered (Figure 3.13).

Normal vehicle treated fetal testes, aged e21.5, contain small clusters of Leydig cells, disseminated evenly throughout the testis. Normally, each cluster occupies <5% of the total Leydig cell area per testis section, resulting in >20 clusters per section. Occasionally, control Leydig cell clusters occupy between 5-15% of the total Leydig cell area per section but clusters >15% were rarely seen in testis sections from control animals. Clusters were categorised by these percentage areas, defined as either small (<5%), medium (5-15%) or large (>15%) (Figure 3.14). The effect of DBP exposure on the distribution of Leydig cell clusters was quantified using computer assisted stereological techniques, detailed in Chapter 2.

Statistical analysis of the data generated showed a significant decrease in the percentage area of the testis occupied by small and medium sized Leydig cell clusters in DBP-exposed testis compared to control testes. The increase in the percentage area occupied by large Leydig cell clusters in DBP-exposed testis was not significant but continued the significant trend away from small clusters. The relevance of this altered distribution pattern was further investigated by comparing steroidogenic output (testosterone content) of control and DBP-exposed testis.

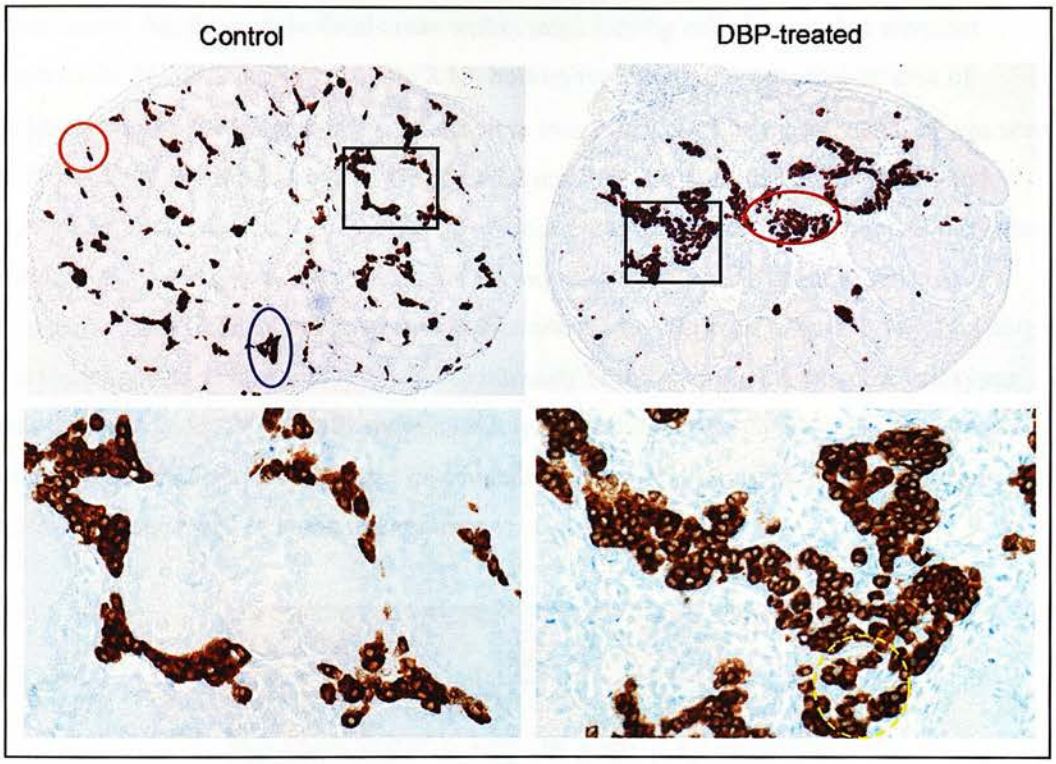

Figure 3.13: 3β-HSD immunostained sections of e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Note the altered Leydig cell clusters after DBP-exposure, compared to the control image. Examples of each cluster category are circled: small (orange), medium (blue) and large (red). A possible region of dysgenesis in a large cluster is highlighted (dashed yellow circle). The top panels were photographed using a x10 objective and highlighted sections (rectangles) were re-photographed using a x100 objective.

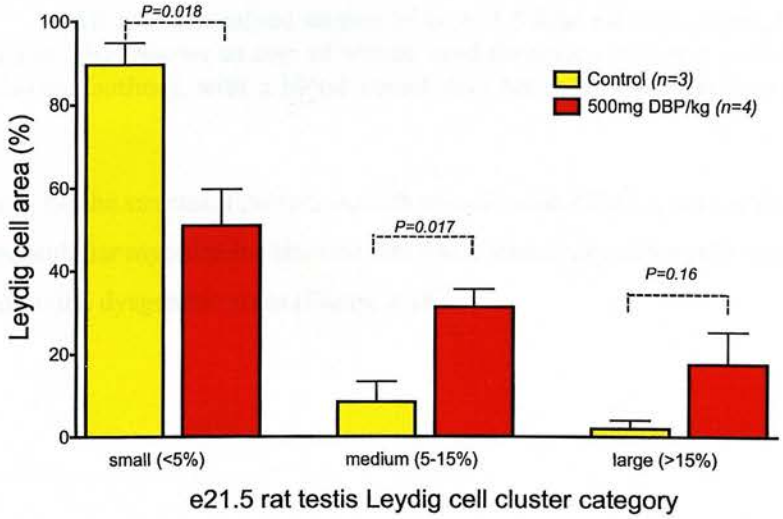

Figure 3.14: Frequency of each category of 3β-HSD immunopositive cluster in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. Data were log transformed prior to statistical analysis.

It was noted that there were focal areas within large Leydig cell clusters that were not positive for 3β-HSD staining (Figure 3.13, bottom right panel), suggesting an area of dysgenesis where non-steroidogenic cells were enveloped, such as Sertoli cells, as was seen with the WT-1 and AMH staining (Figures 3.2 and 3.9). Immunostaining of additional sections for the androgen receptor (AR) protein, which was located in the nuclear membrane of interstitial Leydig cells and peritubular myoid cells, confirmed that most cells in the dysgenetic areas were expressing proteins like normal Leydig cells (Figure 3.15). It is noted that there appears to be a difference in the intensity of the staining between the cell types with the peritubular myoid cells more intensely stained than the Leydig cells. Though the relevance of this difference was not investigated further, it is likely to reflect a difference in the concentration of AR in the membranes.

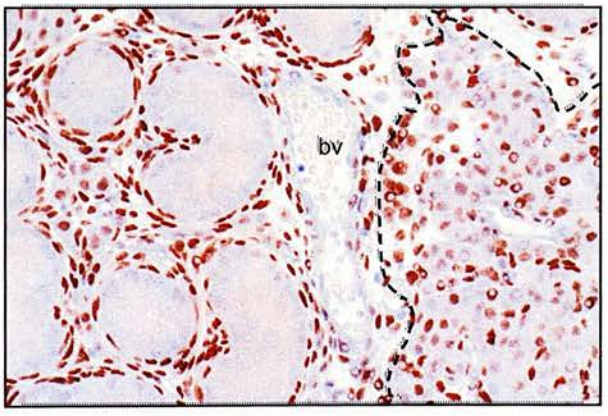

Figure 3.15: AR immunostained section of an e21.5 fetal rat testis after e13.5-e20.5 *in utero* exposure to DBP. Shows an area of normal cord formation next to a possible region of dysgenesis (dashed outline), with a blood vessel (bv) between. Photographed using a x40 objective.

Further staining for the structural protein smooth muscle actin (SMA), unique in the testis cords to the peritubular myoid cells, showed that SMA-immunopositive cells were not widely present in the dysgenetic areas (Figure 3.16).

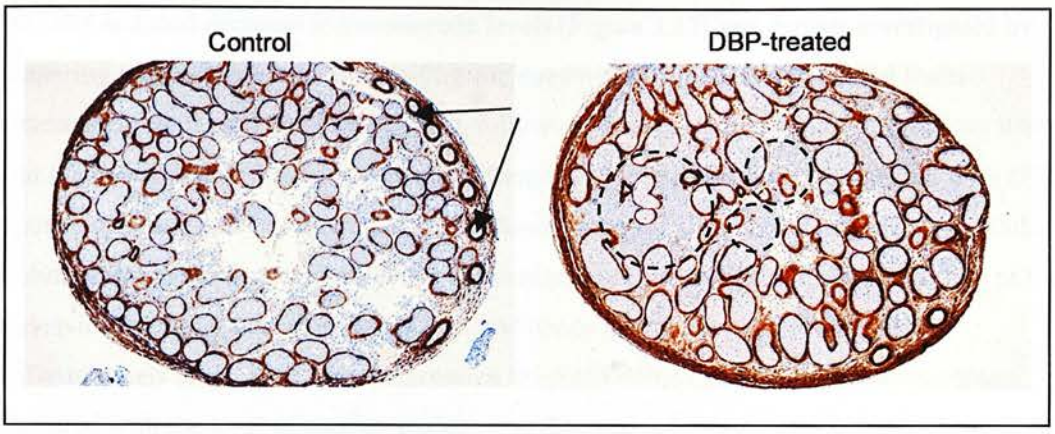

Figure 3.16: SMA immunostained sections of e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Note the abnormal interstitial areas in the treated testis, unstained by SMA (dashed circles). The absence of the distinctive pattern of blood vessels (arrows) circling the perimeter of the treated testis is probably an artefact of sectioning and not of DBP treatment. The irregular cords at the top of the pictures illustrate the developing rete testis region. Photographed using a x10 objective.

The testosterone levels of whole testes per treatment were quantified using a radio-immunoassay (RIA), according to the method detailed in Chapter 2. DBP exposure induced a significant decrease (P=0.047) in testis testosterone levels (Figure 3.17) compared to control testes. The consequences of reduced testosterone levels could be far reaching, given the critical role of testosterone in masculinisation and development of secondary sexual characteristics in the male, and these are discussed at the end of this chapter.

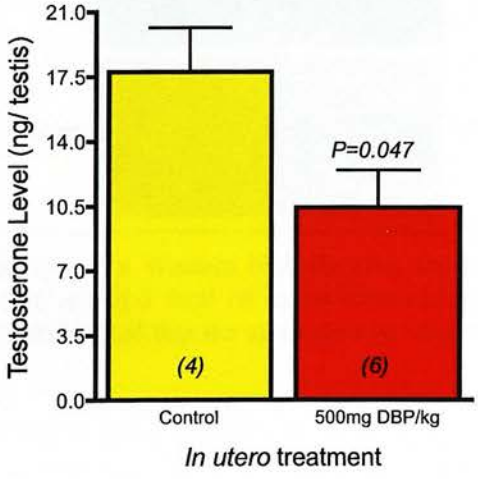

Figure 3.17: Testicular testosterone levels in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). Treated testes had significantly reduced testosterone levels compared to control testes (P=0.047).

The DBP-induced decrease in testosterone levels (Figure 3.17) was further investigated by comparing expression levels of steroidogenic enzymes in treated and untreated testes. Immunostaining for the enzyme 3β-HSD, followed by visual inspection of the sections using light microscopy, had shown no obvious difference in expression levels associated with DBP exposure (Figure 3.13), so alternative steroidogenic targets were considered. The availability of cholesterol to the first enzyme in the steroidogenic cascade (P450scc) limits the rate of the conversion of cholesterol to pregnenolone and hence the rate of steroidogenesis (DiBartolomeis et al., 1987). The expression level of P450scc protein per treatment was compared with the level of another protein, one that was independent of the steroidogenic cascade, a cytoskeletal protein: Smooth muscle actin (SMA). Expression levels of P450scc and SMA were measured and compared per treatment following Western Blot analysis of extracts of total testis protein (Figure 3.18).

DBP treatment showed a highly significant (P=0.0014) decrease in the level of P450scc expression relative to SMA expression when compared to untreated testes (Figure 3.19). This study demonstrated that the rate-limiting step of steroidogenesis was compromised by DBP exposure, suggesting it was a significant target of DBP toxicity in the steroidogenic cascade.

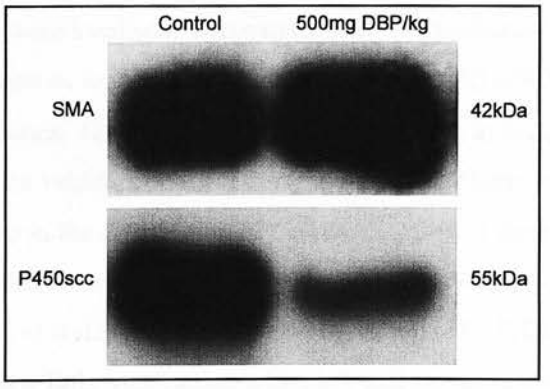

Figure 3.18: Photograph of a Western blot showing the level of P450scc expression relative to SMA expression in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. It is noted that the antibody was very "clean" with no non-specific bands seen on the blot.

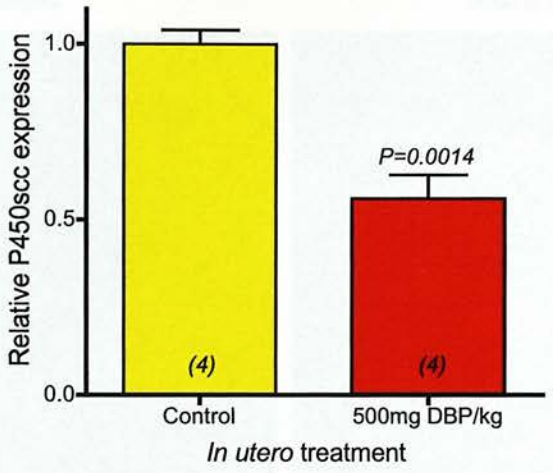

Figure 3.19: The relative amount of P450scc protein expressed by Western blot analysis in e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP was plotted, assuming expression of SMA was 100%. Values are means ± S.E.M. (*n* values are shown in parentheses). The decrease in P450scc expression seen in the DBP treated group was highly significant (P=0.0014).

An additional experiment compared the distribution of P450scc expression against 3β-HSD distribution. This investigated whether P450scc expression levels did vary across the testis or whether any variations were localised. This experiment was facilitated by the use of fluorescent co-localisation of immunostaining of P450scc and 3β-HSD protein expression in Bouin's fixed testis sections. The appearance of co-localisation in the DBP-exposed testis was compared against the vehicle control testis (Figure 3.20). There was no obvious treatment related change in the distribution of the two proteins. If there was a difference, this was proposed to be evident as a shift from away from green (P450scc), reducing the amount of yellow (co-localisation) and increasing the amount of red (3β-HSD) staining seen. The sections appeared to show little localisation with each cell expressing either of the two proteins but not both, in a treatment independent fashion. Expression levels were not quantified but qualitative assessment suggested that P450scc expression levels were varied across control and treated clusters (Figure 3.20). This suggested that the reduction in P450scc expression seen with the Western blot analysis (Figure 3.18) was not localised but was more likely to be evenly distributed across the DBP-treated Leydig cells.

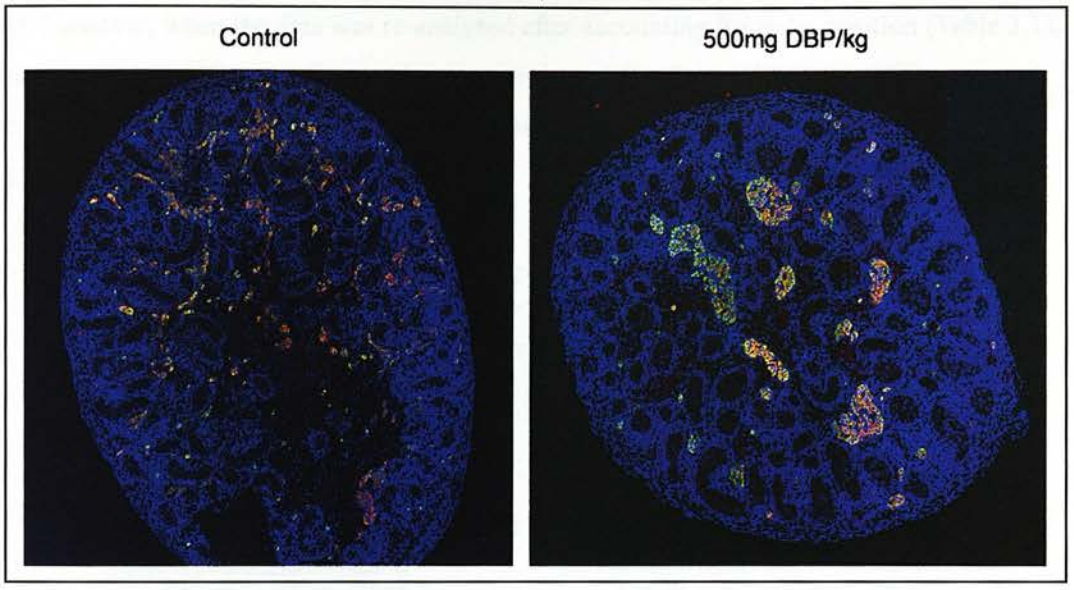

Figure 3.20: Confocal analysis of immunostained sections of e21.5 fetal rat testes after e13.5-e20.5 *in utero* exposure to vehicle (control) or DBP. Sections were immunostained for 3β-HSD (red) and P450scc (green) with co-localisation seen as yellow. Blue = nuclear counterstain. There was no obvious effect of treatment on the co-localisation of the two proteins. The altered Leydig cell distribution with DBP treatment is evidenced in the right hand panel by the reduced number but larger size of Leydig cell clusters. Tiled images (7x7), each tile captured using a x40 objective.

Overall, the histology of all three of the major cell types in the fetal testis was affected at e21.5 after e13.5-e20.5 *in utero* exposure to 500mg DBP/kg. The long-term consequences of the DBP induced changes seen at e21.5, were followed up by observing adults rats, following e13.5-e21.5 *in utero* exposure to 500mg DBP/kg.

3.3.2 Effect of fetal exposure to DBP on the adult testis

The previous studies investigated the effect of 500mg DBP/kg *in utero* exposure on the fetal testis at e21.5. To follow-up the changes induced by DBP on fetal testis development, fecundity, testis descent and plasma hormone levels were compared in adult animals, left to mature under control conditions to at least 90 days of age, after *in utero* exposure to DBP or the vehicle control. It may have been interesting to generate untreated animals with cryptorchidism but this was not possible under the project license or with in-house surgical skill base available.

Adult testis weights were measured and their position recorded at necropsy, prior to fixation. The mean weight of testes in adult animals exposed *in utero* to 500mg DBP/kg was significantly reduced compared to age matched vehicle control animals (Figure 3.21, panel

A). However, when the data was re-analysed after accounting for testis position (Table 3.1), there was no significant difference in mean testis weight of scrotal testes ± DBP treatment, but an increase in the significance between the Control (scrotal) testes and the DBP-treated cryptorchid testes (Figure 3.21, panel B).

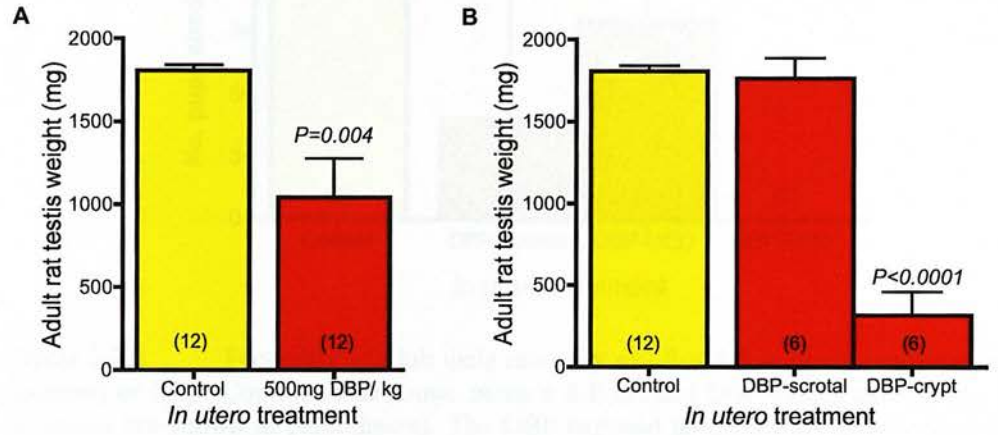

Figure 3.21: Adult testis weight per rat after e13.5-e21.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. (*n* values are shown in parentheses). The DBP exposed group showed a significant (P=0.004) decrease in adult testis weight when all data was compared to the testes from control animals (panel A). There was no significant difference in mean weight of scrotal testes ± DBP exposure, but a highly significant difference in the mean weights of DBP exposed cryptorchid testes (P<0.0001) (panel B) compared to Control testes.

Six animals from each treatment group were paired with an untreated but proven female, until a positive sign of mating (plug) was detected. All of the control animals sired a litter of at least 11 pups, but only 50% of the DBP exposed adults sired any pups (Table 3.1, Figure 3.22).

In utero treatment (e13.5-e21.5)	Cryptorchidism incidence at>90d		Number of pups sired at>90d	Fecundity incidence at>90d
Vehicle control *(n=6)*	normal	6	>10	6
	unilateral	0	<10	0
	bilateral	0	(infertile)	0
500mg DBP/kg *(n=6)*	normal	2	>10	1
	unilateral	2	<10	2
	bilateral	2	(infertile)	3

Table 3.1: Summary of testis position and fecundity in adult male rats (>90d) after *in utero* exposure to vehicle control or 500mg DBP/kg over e13.5-e21.5. Testis position was recorded at necropsy.

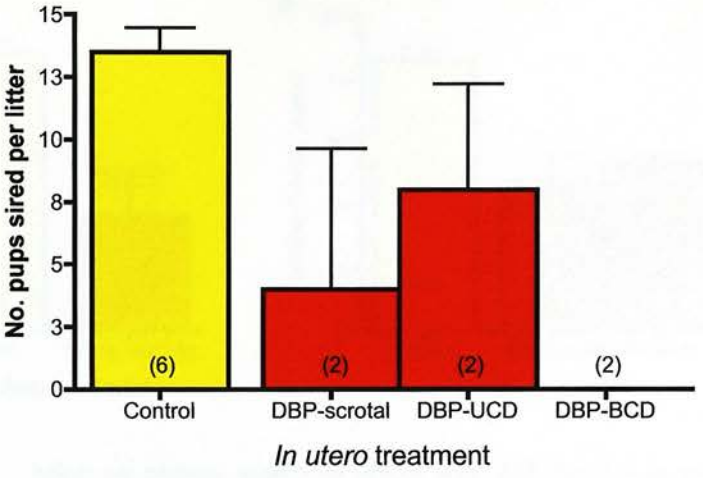

Figure 3.22: Fecundity of adult male rats after e13.5-e21.5 *in utero* exposure to vehicle (control) or DBP. Control value shows mean ± S.E.M. and DBP values show mean ± range (*n* values are shown in parentheses). The DBP exposed animals were sub-divided by testis position: scrotal (normal), unilaterally cryptorchid (UCD) or bilaterally cryptorchid (BCD). All DBP exposed animals sired less pups than the control animals, regardless of testis position.

The level of two hormones was measured in plasma collected at necropsy. Inhibin-B and testosterone were quantified using methods described previously in Chapter 2. The level of inhibin-B was significantly reduced in the plasma of all DBP exposed animals, but was further reduced in bilaterally cryptorchid animals compared to those with scrotal testes or unilateral cryptorchidism (Figure 3.23). The reduced inhibin-B level in the treated animals was paralleled by reduced fecundity (Figure 3.22). There appeared to be an inhibin-B cut-off point, around 120ng/ml, below which the males were infertile, regardless of their testis position. This data showed 1/6 animals exposed to DBP did have a plasma inhibin-B level and litter size within the control range, albeit at the lowest end. There was no significant effect of *in utero* DBP treatment on adult plasma testosterone levels (Figure 3.25) and no correlation between testosterone levels and fecundity (Figure 3.26).

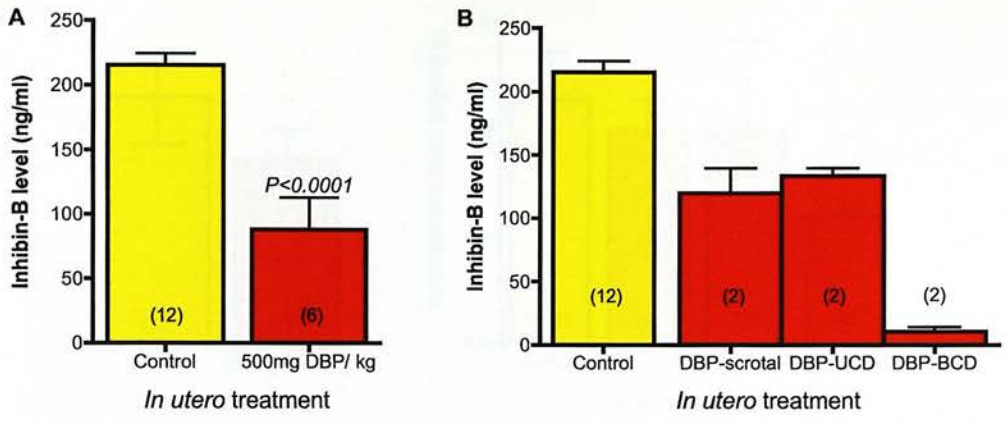

Figure 3.23: Adult rat plasma inhibin-B levels after e13.5-e21.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. unless n=2 when values show mean ± range (*n* values are shown in parentheses). In panel A, the DBP exposed group showed a significant (P<0.0001) decrease in inhibin-B level compared to the testes from animals exposed to vehicle only. When the DBP exposed animals were sub-divided by testis position: scrotal (normal), unilaterally cryptorchid (UCD) or bilaterally cryptorchid (BCD), all treated animals showed a decrease in their inhibin-B level, with the most dramatic reduction in BCD animals.

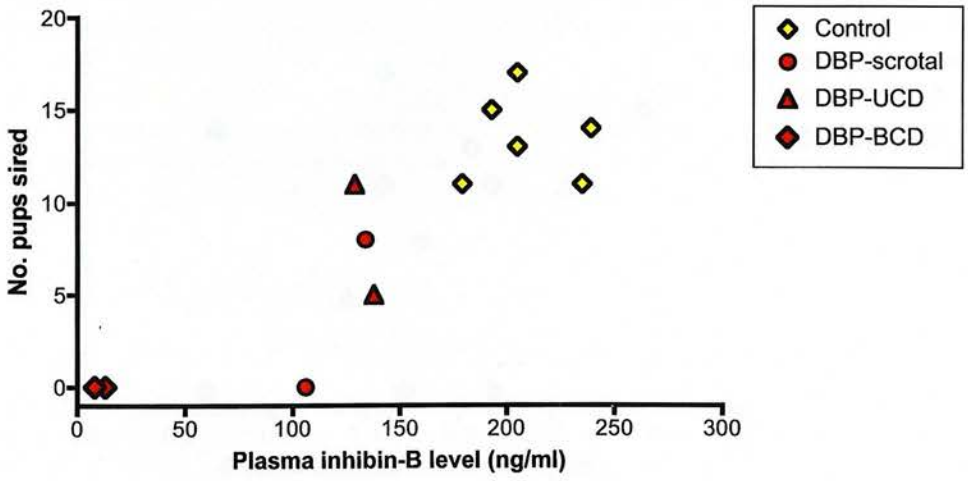

Figure 3.24: Scatter plot comparing plasma inhibin-B levels with number of pups sired by adult rats after long-term *in utero* exposure to control vehicle or DBP. Individual data are presented (n = 6 for both groups). The DBP exposed group showed lower inhibin-B levels and fewer pups sired, if any, compared to the vehicle-exposed adults.

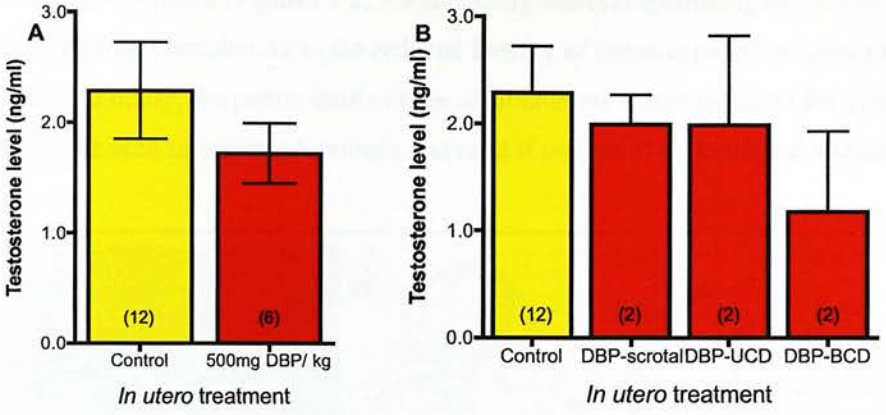

Figure 3.25: Adult rat plasma testosterone levels after e13.5-e21.5 *in utero* exposure to vehicle (control) or DBP. Values are means ± S.E.M. unless n=2 when values show mean ± range (*n* values are shown in parentheses). In panel A, the DBP exposed group showed a non- significant decrease in testosterone level compared to animals exposed to vehicle only. When the DBP exposed animals were sub-divided by testis position: scrotal (normal), unilaterally cryptorchid (UCD) or bilaterally cryptorchid (BCD), all treated animals showed a decrease in their testosterone level, with the most dramatic reduction in BCD animals (panel B).

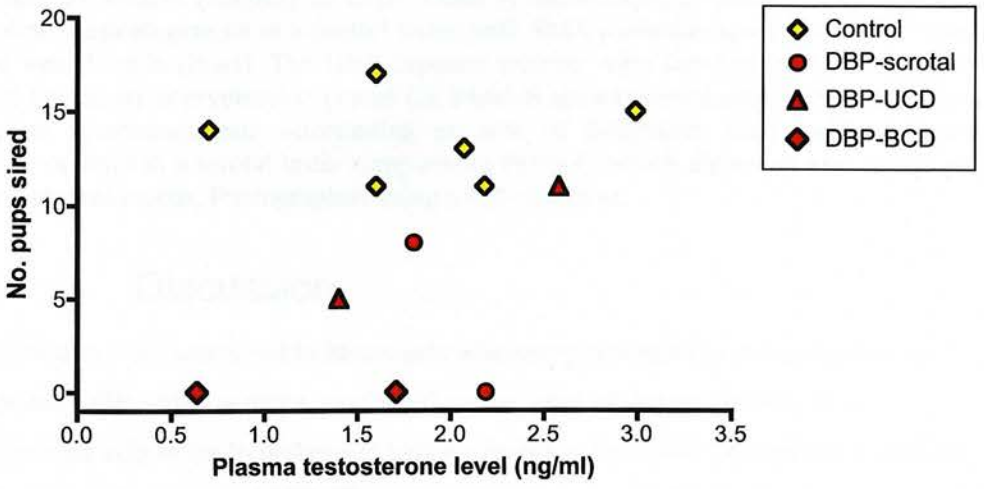

Figure 3.26: Scatter plot comparing plasma testosterone levels with number of pups sired by adult rats after long-term *in utero* exposure to control vehicle or DBP. Individual data is presented (n = 6 for both groups). The DBP-exposed animals were sub-divided by testis position: scrotal (normal), unilaterally cryptorchid (UCD) or bilaterally cryptorchid (BCD). The treated rats showed similar testosterone levels but fewer pups sired compared to the controls.

Low magnification review of SMA stained sections of adult testis showed abnormal staining patterns in scrotal and cryptorchid testes from DBP-exposed animals (Figure 3.27). These focal areas of tubule malformation were consistent with the dysgenetic areas seen in the

e21.5 testis sections (Figures 3.2, 3.9 and 3.13). Normal spermatogenesis was not apparent in these regions, contributing to the reduced fertility of testes exposed *in utero* to 500mg DBP/kg. Finally, the penile shafts of the adult animals were examined for hypospadias. This was never seen in untreated animals and mild if present at all in treated animals.

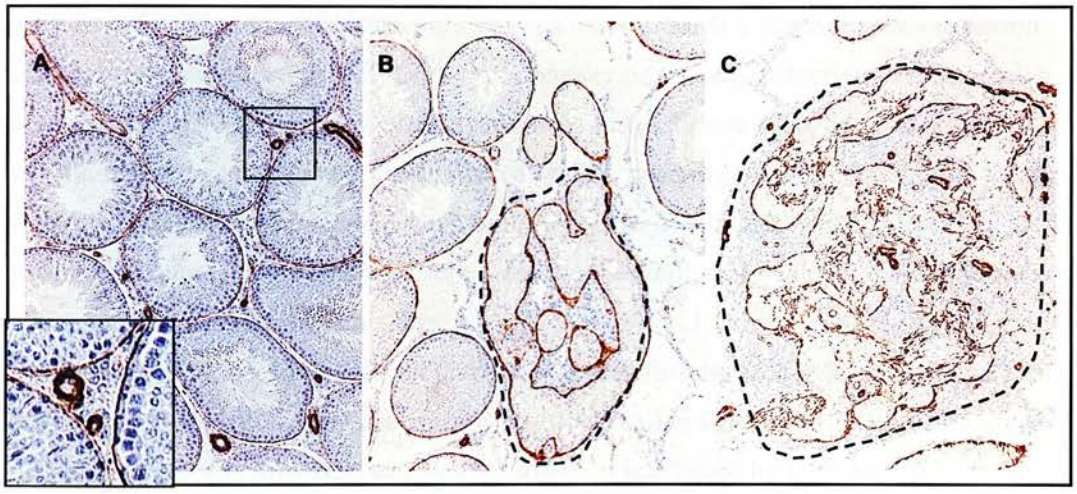

Figure 3.27: SMA immunostained sections of adult rat testes after e13.5-e21.5 in utero exposure to vehicle (control) or DBP. Panel A shows normal seminiferous tubules with complete spermatogenesis in a control testis, with SMA immunostained peritubular cells and blood vessel walls (inset). The DBP exposed animals were sub-divided by testis position: scrotal (Panel B) or cryptorchid (Panel C). Panel B shows normal seminiferous tubules with complete spermatogenesis surrounding an area of dysgenesis with malformed tubules (dashed outline) in a scrotal testis compared to Panel C, which shows an area of dysgenesis in a cryptorchid testis. Photographed using a x10 objective.

3.4 Discussion

These studies were conceived to investigate whether exposing male rats to the synthetic compound DBP whilst *in utero*, would induce the array of changes in their testis development akin to the hypothesised human condition Testicular Dysgenesis Syndrome (TDS) (Skakkebaek et al., 2001). The successful development of an animal model for this syndrome would provide scientists with a means to procure a better understanding of the fetal origins and adult onset of the conditions. To this end, the gross histology of the rat testis and its major cell types were compared between rat pups at e21.5 after exposure to 500mgDBP/kg/ day over e13.5-e20.5 or adult rats at >90d after exposure to 500mgDBP/kg/ day over e13.5-e21.5 with their respective vehicle exposed controls.

In TDS, the most common finding is reduced sperm count in adulthood, with up to 30% of young Danish men affected (Jensen et al., 2002). It is well known that a decrease in sperm numbers in adults can result from a reduction in Sertoli cell numbers (Orth, 1982; Orth,

1984). In the present studies neither inhibin-B production nor the Sertoli cell size, number or rates of proliferation and apoptosis in rat testes at e21.5 were affected by in utero exposure to DBP but there was a decrease in the volume of the testis occupied by Sertoli cells (P=0.047). This suggests that though the proliferation index (%) was not significantly affected by treatment with DBP in the present studies, the fall from a PI% of 35.9% to in the control animals to 29.5% in treated testes, did suggest a trend towards a slight decrease in Sertoli cell proliferation following DBP exposure. Considering that any reduction in Sertoli cell numbers due to decreased proliferation prior to puberty can result in a decreased testis weight, reduced sperm numbers and reduced fertility in adults, this observation was followed up in adult rats following *in utero* DBP exposure (Orth, 1982; Orth, 1984). The effect of this treatment regime on the number of Sertoli cells in the adult testis is under investigation by other members of this research group.

The level of inhibin-B, a Sertoli cell product, was significantly reduced in the plasma of all DBP exposed animals, regardless of testis position. Inhibin-B is used clinically as a marker of spermatogenesis (Anderson and Sharpe, 2000). It is affected by testis position as seen by the analysis of human plasma for inhibin-B from a group of men with scrotal testes who had a significantly ($P<0.01$) higher median level of inhibin-B (144ng/l) than did cryptorchid patients (98ng/l) (de Gouveia Brazao et al., 2003). In the present studies, two thirds of the DBP exposed rats had cryptorchid testes at necropsy. The level of plasma inhibin-B was further reduced in bilaterally cryptorchid animals compared to those affected unilaterally. There was an inhibin-B cut-off point (around 120ng/ml serum) below which the males were infertile, regardless of their testis position and all adult rats exposed to DBP *in utero* showed a dramatic decrease in their fecundity as seen by the lower number of pups they sired compared to vehicle-exposed controls.

Additional work carried out in this laboratory further investigated the effect of *in utero* DBP exposure on the maturation of the Sertoli cell using immunohistochemistry (Fisher et al., 2003). Three protein markers were used: Wt-1 as expressed by Sertoli cell nuclei at all ages, AMH as expressed in immature Sertoli cell cytoplasm and $p27^{kip}$ seen in nuclei of differentiated Sertoli cells. The results showed that $p27^{kip}$ was notably absent in abnormal tubules in adulthood such as those without germ cells or adjacent to dysgenetic areas, though $p27^{kip}$ was present in normal tubules of scrotal testes of DBP exposed rats at d25 postnatal and in adulthood.

Together, these data suggest that Sertoli cells can be permanently damaged following *in utero* exposure to DBP, consistent with the reduced fertility phenotype of the adult animals and the low sperm counts of TDS.

The high incidence of adult cryptorchidism in the DBP-exposed rats of the present studies is consistent with the significant decrease in testosterone measured in treated testes at e21.5. Cryptorchidism describes the maldescent of the testis and is a two-phase process, of which the second, inguino/scrotal, phase is androgen dependent (Ivell and Hartung, 2003). The first phase involves the development of the gubernaculum and the regression of the cranial suspensory ligament, pulling the fetal testis down the abdominal cavity ready for inguino/scrotal descent. In a recent study using the same DBP-exposure regime as the present studies, DBP exposure suppressed *Insl-3* protein expression in fetal Leydig cells but no effect was seen in testes exposed to the androgen receptor antagonist flutamide, suggesting that DBP has an androgen independent effect on fetal testis descent (McKinnell et al., 2005).

Cryptorchidism is associated with abnormalities in other androgen dependent organs such as hypospadias as androgens are required for the formation of the penile urethra and the scrotum from the bipotential genital tubercle (Klonisch et al., 2004). Disruption in the levels of androgens during this phase of development can result in malformation of the penis such as hypospadias, the malpositioning of the penile urethra. In these studies, the presence of mild hypospadias was noted in adult rats exposed to DBP *in utero*.

Together, these data suggest that secondary sexual phenotype including testis position and penile shaft development can be permanently damaged following *in utero* exposure to DBP, producing a phenotype in the adult animals consistent with the cryptorchidism and hypospadias that form part of the profile of TDS.

Similar experiments to those presented here were carried out by a different laboratory and using a different strain of rats and are reported by Barlow *et al* (2004). Time-mated Sprague-Dawley dams were treated by gavage with 500mg DBP/kg/day from e12-e21 and the male pups necropsied at 6, 12 or 18 months of age. The gross lesions of the reproductive tract induced in adult animals exposed to DBP *in utero* were similar to those described in the present studies. Additional observations of a decreased anogenital distance (AGD) on postnatal day 1 (d1) and increased retention of areolae on d13 were reported as well (Barlow et al., 2004). Neither AGD nor areolae retention were assessed for the animals in the present studies as they are not part of the TDS profile of disorders, but their maldevelopment is indicative of disturbed fetal androgen levels (McIntyre et al., 2001). The present studies extended the observations of gross malformations of the reproductive tract induced by *in utero* DBP exposure, to include immunohistochemistry studies of the androgen producing cells of the fetal testis.

Analysis of the distribution of the Leydig cells, using immunohistochemical staining for the steroidogenic enzyme 3β-HSD, indicated that *in utero* DBP exposure significantly altered the distribution of the Leydig cells within the developing testis at e21.5, inducing the formation of fewer but larger clusters of Leydig cells. Abnormal aggregation of human Leydig cells into clusters, termed micronodules, has been reported to be more frequent in testes with impaired spermatogenesis and thus a histological marker of testicular failure in man (Holm et al., 2003).

Additional work in this laboratory (M.Walker) showed that there was no significant change in the actual number of fetal Leydig cells (around 150000 per testis) or their rate of proliferation at e21.5, associated with DBP exposure. However, the cytoplasmic volume of the treated Leydig cells was significantly reduced (Mahood et al., 2005). The adverse impact of this change was reflected in the highly significant decrease in testicular testosterone measured in DBP exposed testes at e21.5. Considering that steroidogenesis occurs in Leydig cell mitochondria and cytoplasm, further study was made into the expression levels of P450scc, the enzyme responsible for the conversion of cholesterol into the androgen precursor pregnenolone. The highly significant decrease in P450scc expression but lack of effect on 3β-HSD expression measured in exposed e21.5 testes suggested a highly selective mechanism of toxicity was induced by DBP exposure, worthy of further and detailed investigation.

As well as disrupting the production of hormones by the fetal testis, the DBP exposure regime described in the present studies also upset the compartmentalisation of the developing testis, creating areas of dysgenesis. Immunostaining of the peritubular myoid cells in adult testes following *in utero* DBP exposure, revealed regions of irregular tubules with an inappropriate mixture of cell types compared to normal testicular architecture. Further study into the ontogeny of the formation of these areas, was carried out by other members of this laboratory. Dysgenetic areas as such were not evident in the fetal testes of DBP-exposed animals but Sertoli cells and peritubular myoid cells were detected within the abnormal Leydig cell aggregates (Fisher et al., 2003; Mahood et al., 2005). By d4, these aggregates appeared to be re-organising into misshapen seminiferous cords that also contained Leydig cells, relocating them to an intra-tubular position along with germ cells. By adulthood, tubules with intra-tubular Leydig cells had lost their germ cell complement. Not all tubules without germ cells had obvious intra-tubular Leydig cells and these Sertoli cell only (SCO) tubules were often situated alongside the dysgenetic regions. Focal areas of dysgenesis were found in adult testes exposed in utero to DBP regardless of whether the testes were scrotal or cryptorchid, though the scale of abnormality appeared to reflect the

level of cryptorchidism (Mahood et al., 2005). The development of dysgenetic areas reduces the capacity of the adult testis for normal spermatogenesis, and presumably lowers the sperm count output.

Dysgenetic features, including immature Sertoli cells and multiple SCO tubules as well as immature or morphologically distorted tubules, have been reported in human testes contralateral to those with a diagnosed germ cell tumour (Hoei-Hansen et al., 2003). Additionally, the biopsies revealed the presence of calcium deposits within the seminiferous epithelium, termed microliths (Skakkebaek et al., 2003). These formations can be detected non-invasively using ultrasound and have been shown to be an indicator of germ cell cancer because of their association with carcinoma *in situ* (CIS) cells (Kim et al., 2003). This association in the testes of adult rats following *in utero* DBP exposure cannot be investigated, due to the absence of CIS cells in rat testes.

CIS cells are believed to originate from fetal germ cells and though are non-invasive in them selves (they differentiate into invasive cell types), they are associated with the majority of germ cell tumours, the most common type of testis tumour in man. CIS cells are used as an indicator of dysgenesis in human adult testes and are even found in up to 9% of biopsies from testes contralateral to those with a diagnosed germ cell tumour (Hoei-Hansen et al., 2003). CIS cells in human testes can be detected following immunostaining for PLAP (Placental alkaline phosphatase) but this antibody does not work in the fetal or adult rat testis. However, the appearance of abnormal germ cells in fetal rat testes (multinucleated gonocytes) and the degeneration of the adult seminiferous epithelium into SCOs following *in utero* DBP exposure are described in the present studies and reported by others (Barlow et al., 2004; Fisher et al., 2003; Mahood et al., 2005).

Overall, the aim of these studies was to test whether the profile of abnormalities that make up the hypothesised Testicular Dysgenesis Syndrome could be reproduced in the male rat following its *in utero* exposure to 500mg DBP/kg/day over e13-21. Three of the four gross disorders of the syndrome were induced in the rat, following the disturbance of fetal testis development by DBP. This data supports the hypothesis outlined by Skakkebaek *et al* (2001) that TDS is the result of disruption of gonadal development during fetal life.

Of the changes reported in the dysgenetic human testis, SCO tubules and immature Sertoli cells, abnormal Leydig cell aggregation (micronodules) and dysgenetic areas were also seen in the present rat DBP model. CIS cells and microliths were not observed in the present animal studies, nor have these findings been reported in adult rat testes by other laboratories following this treatment regime. However, this regime did induce abnormal fetal germ cells and areas of germ cell depleted tubules in the adult rat testis. It may be that testis germ cell

cancer does not have a comparable fetal origin between the rat and the human. This outcome was not seen as a limitation of the model as it should be noted that low sperm count, cryptorchidism and hypospadias are all individual risk factors for the development of testicular cancer and were all induced by this treatment regime (Sharpe, 2001). Therefore it is believed that detailed characterisation of the mechanisms through which DBP induces its repertoire of effects are probably of direct relevance to studies of human TDS (Skakkebaek et al., 2001). By understanding how particular chemicals induce TDS in rats, it might become possible to identify comparable mechanisms and potential causes in humans.

To this end, novel *in vitro* experiments were conceived using explants of fetal testes. *In vitro* studies would allow time- and dose- controlled exposure to the treatment, applied directly to the testis and help towards the elucidation of the mechanism behind DBP toxicity. Following rigorous optimisation of the novel *in vitro* system, explants were exposed to the DBP metabolite MBP and the resulting changes in testis architecture and hormone production were compared. The data generated from explants with fetal rat testes are reported in Chapter 4 and data from explants of fetal human testes are reported in Chapter 5. Further investigations into the effects on fetal rats testis morphology and steroidogenesis of acute DBP exposure are described in Chapter 6. An overall discussion of the role of this model in elucidating the possible environmental causes behind the increasing levels of TDS and current theories regarding the mechanism of toxicity employed by DBP are described at the end of this thesis in Chapter 7.

4 Studies using fetal testis explants from the rat

The previous chapter demonstrated that in utero exposure of the rat fetus to doses of 500mg DBP/kg/day from embryonic day 13 (e13) induced reproducible phenotypic changes in the fetal testis. It was therefore investigated which, if any of the changes could be induced in fetal testes using an in vitro system of exposure. In vitro studies would allow time- and dose-controlled exposure to the treatment, applied directly to the testis and help towards the breakdown of the phthalate mechanism of action. The experiments described in this chapter extend the analysis of the actions of DBP on fetal testis development seen with the in vivo model and examine the effect of this treatment during development in vitro.

4.1 Introduction

In these studies, an organotypic culture experimental approach was proposed to assess any effects of the DBP metabolite MBP, on the in vitro production of hormones and the development of the testicular somatic and germ cells. This approach provided a unique method to study testis development while largely maintaining normal structural relationships amongst the different testicular cell types. To investigate whether MBP might induce similar affects in the human, parallel studies were carried out using second trimester fetal human testes collected from legally terminated pregnancies. These experiments are described in Chapter 5.

At the time when these studies were conceived, the range of published work that described the effects of DBP on fetal rats, largely examined the adverse effects of phthalate administration on fetal rats via maternal exposure. There was little work describing ex vivo fetal testis exposure to phthalates though this exposure route had been used to investigate other test compounds and their physiological effects on the growth and development of the fetal testis in vitro. These studies formed the background to the experiments described in this Chapter.

4.1.1 The in vitro approach

The very nature of the in vitro approach meant that the developing testes would be removed from their natural environment and blood supply, in the body cavity. However, studies using ex vivo aggregates of fetal testis tissue (e12-20) showed they could re-form into recognisable testicular tissue after at least 24h in culture (Grund et al., 1986). These observations supported studies with decapitated fetuses which had no access to pituitary products but underwent normal testis cord formation (Habert and Picon, 1982). Similarly, transgenic mice reported by Kendall et al (1996), were devoid of the biologically active forms of the gonadotrophins LH, FSH and thyroid stimulating hormone (TSH), yet, fetal and neonatal

development of the genital structures of both sexes was normal. More recently, studies looking at the ontogeny of plasma LH levels and steroidogenesis in the rat fetal testis, confirmed that basal testosterone production rises from e16.5 to a peak between e18.5-e19.5, though plasma LH levels were almost undetectable until e19.5 but increased significantly thereafter (El-Gehani et al., 1998). Together these experiments confirm that fetal testis development and testosterone production in the rodent must occur normally with very low if any gonadotrophin influence.

Unlike a dispersed cell culture system, the organ culture system preserves testicular architecture including the many and varied intercellular communications. The organotypic system has the advantage of not dispersing secreted factors immediately into the media as in a cell culture system but enables the secreted factors to influence the adjacent testicular cells to develop as if in vivo (Livera et al., 2000). This approach has been successfully applied to experiments investigating the development of fetal testes which would form the basis for this optimised system (Habert et al., 1991; Lecerf et al., 1993; Livera et al., 2000).

Experiments carried out by Richards et al (1999), highlighted that culture conditions optimised for one set of cells may not be optimal for another similar set or even the same cells at a different developmental stage. In their case, murine migratory primordial germ cells required different conditions than did the older gonadal germ cells (Richards, 1999). This emphasised the importance of culture condition optimisation.

4.1.2 Endocrine disruptors and DBP in vitro

Studies have shown that even at low levels of exposure, at micromolar (μM) concentrations, synthetic endocrine disruptor chemicals can induce changes in the architecture of the developing testis. For example, methoxychlor (an alternative to the banned pesticide DDT) is biotransformed in the liver to two potent metabolites; of these, HPTE was proposed to be weakly oestrogenic as it binds to the oestrogen receptor more strongly than does the parent compound (Akingbemi et al., 2000). HPTE and methoxychlor were assessed in vitro to investigate their effect on embryonic rat testis cord formation (Cupp and Skinner, 2001). E13.5 testes cultured ± the two compounds for 3 days, at doses of up to 20μM, showed treatment related alterations in the growth and proliferation pattern of the cells within the developing testis cords. In another study, the in vivo and in vitro oestrogenic activities of eight phthalate esters were compared (Zacharewski et al., 1998). DBP demonstrated weak oestrogen receptor mediated activity at high concentrations (10μM) in vitro but no significant estrogenic effects were observed in vivo (up to 2000mg/kg). Effects of DBP on whole e9.5 rat embryo cultures at dose levels from 1μg/ml to 1mg/ml, exposed for 48h, were

reported by Rhee et al (2002). Along with two other phthalates (DEHP and BBP), this short-term in vitro assay demonstrated that all three phthalates inhibited growth in a dose-dependent manner. At high concentrations, all three phthalates had an adverse effect on whole embryo growth and development including induction of severe head abnormalities, short tails and short limbs. In further tests with limb bud and mid-brain cell cultures, DBP was found to exhibit the most embryotoxic potential. The authors went on to say that with phthalates, as with many other chemical classes, the toxicity can vary with chemical specification (Rhee, 2002).

4.1.3 Experimental Objectives

The aim of these studies was to set up a culture system that allowed normal fetal testis development to occur autonomously in vitro, and then to investigate whether that normal development was disrupted with in vitro exposure to DBP. For that purpose, the development of the testis explants was assessed using 3 main criteria:

- maintenance of tissue architecture and protein expression
- analysis of the somatic cell proliferation rate
- measurement of hormone production ± stimulation/ inhibition

The system would be considered optimised when measurements of the three criteria showed minimal differences between in vitro cultured explants and the expected development in vivo over time. Only then would phthalates be introduced as a treatment for comparison. The three criteria set for optimisation would also be used to compare the endpoints seen in the testes exposed to DBP in utero with any seen in the explants exposed in vitro.
These studies were designed to investigate the effects of the DBP metabolite MBP on testis growth and development through evaluation of hormone production and morphology in cultured fetal rat testis explants. Response to treatment involved comparison of histology and hormone production in explants cultured in treated media with other explants from the same incubation experiment cultured in untreated media, using an optimised organotypic culture system.

4.2 Methods and Materials

Further details on the general methods and materials used are listed in Chapter 2. Overall, following completion of the incubation, media were stored at –20°C before being assayed for hormone production and explants were analysed for histological changes.

4.2.1 Explant preparation

Fetal rat testes were collected and explants prepared as detailed in Chapter 2. Testis explants were cultured on a 0.45μm pore size culture insert with tripod scaffold (Millipore, UK),

secured in a well of a 24-well tissue culture plate, as previously described (Habert et al., 1991). Up to six explants were placed carefully on the filter and covered in 0.2ml media. A further 0.2ml of media were placed between the well and the scaffold. Explants were incubated at 37°C in an atmosphere of 5% CO_2 for up to 72h. Media were not changed during the experiment but collected at the end and stored at –20°C until assayed. At the end of the culture period, tissue was preserved, either by chemical fixation in Bouin's fixative or by freezing for storage at –80°C and media were preserved by freezing for storage at –20°C.

4.2.2 Explant culture conditions

4.2.2.1 Optimisation

To establish optimal in vitro culture conditions for the fetal testis explants, various incubation durations, media, media supplements and developmental ages were investigated. All incubations were carried out at 37°C, in a humidified atmosphere of 95% air/5% CO_2. The culture conditions that enabled the explants to maintain the most in vivo-like development were used for extended experiments. The starting point for the incubation media were a comparison of two methods already established in house, though for the incubation of non-testicular tissue.

	Regime A Human fetal gonads	Regime B Rat perinatal prostate	Regime C Rat fetal testis
Basic Media	Alpha MEM	DMEM/ F12	DMEM/ F12
Media Supplements	L-Glutamine Sodium Pyruvate ITS BSA	Transferrin (Bovine) Insulin - -	L-Glutamine Sodium Pyruvate ITS BSA
Anti-microbial products	Penicillin/ Streptomycin Amphotericin	Penicillin/ Streptomycin Amphotericin	Penicillin/ Streptomycin Amphotericin

Table 4.1: Summary of media and supplements used with the three media regimes, used for in vitro incubation of fetal rat testes. Further details are listed in Chapter 2.

4.2.2.2 In vitro treatments

Following selection of the most optimal culture regime, extended experiments were performed with various treatments added in vitro. The treatments were mixed with the culture media prior to addition to the culture well and incubation of the testis explants. The treatments used, their rationale, preparation and dose levels, are detailed in Chapter 2.

4.2.3 Testis architecture and protein expression

Up to six explants were incubated on a single filter, in a single well. Explants from the same well were fixed and embedded together in the same paraffin block. Each block was serially

sectioned (5μm slices), mounted on numbered slides then stained and subjected to analysis as a single replicate. Explants that were fixed in Bouin's were examined microscopically after immunohistological processing. Biological markers were used to reveal the explant morphology. Markers used in these experiments are detailed below.

4.2.3.1 Distribution of peritubular myoid cells

Formation of the seminiferous cord requires peritubular myoid cells (Cupp et al., 2003). These can be visualised using immunostaining for the cytoskeletal protein Smooth Muscle Actin (SMA). One slide per block was stained and analysed at low magnification. SMA distribution between treatments was compared.

4.2.3.2 Quantification of Leydig cell distribution

Three numbered slides per block (at least five serial sections apart) were deparaffinised, rehydrated and immunostained for 3β-HSD using a mouse anti-3β-HSD monoclonal antibody (gift, Professor JI Mason, University of Edinburgh) and lightly counterstained with haematoxylin, according to the protocol described in Chapter 2. The total area of stained tissue per slide was measured and the distribution and size of brown clusters (immunopositive Leydig cells) within the outlined blue (counterstained) area was quantified, using a light microscope and a x63 objective and computer-assisted image analysis as described in detail in Chapter 2 and similar to that published (Mahood, et al. 2005). At least four blocks per treatment were analysed.

4.2.3.3 Quantification of multinucleated gonocytes

One slide per block, the same numbered section per block, was deparaffinised, rehydrated and immunostained for AMH using a goat anti-AMH polyclonal antibody (Santa Cruz) and counterstained with haematoxylin, according to the protocol described in Chapter 2. The large spherical nuclei of the gonocytes were easy to distinguish, amongst the AMH stained Sertoli cell cytoplasm, under a light microscope at x63 magnification, as were the counterstained perimeters of the nuclear membrane(s). The number of seminiferous cords per cross section was counted and each cord assessed for containing none or at least one gonocyte with >1 nuclei per cytoplamsic membrane (a multinucleated gonocyte). The percentage of cords containing at least one multinucleated gonocyte (MNG) was calculated per block. At least ten blocks per treatment were analysed.

4.2.4 Analysis of the somatic cell proliferation rate

During fetal testis development, the Sertoli cells are proliferating. The rate of proliferation can be measured by exposing the testes to the nucleotide base dUTP, supplied in vitro as the parent compound 5-Bromo-2'-deoxyuridine (BrdU), which is incorporated into the

reproduced DNA in place of the dTTP base. The incorporation of the dUTP base can be visualised by immunostaining with a relevant antibody. In order to ensure that proliferation was quantified in Sertoli cells and not another testis cell type undergoing proliferation, sections were double immunostained for BrdU and the Sertoli cell nuclear specific marker WT-1. WT-1 staining was visualised with Fast blue which co-localised with the brown DAB developed BrdU antibody, together they uniquely identified proliferating Sertoli cell nuclei. The incidence of Sertoli cell apoptosis was also investigated by labelling fragmenting (apoptotic) DNA by catalytic incorporation of fluorescein-12-dUTP at the 3'-OH ends of the DNA using the terminal deoxynucleotidyl transferase enzyme. This method could discern apoptotic cells from cells undergoing necrosis, also recognisable microscopically by their darkly counterstained pyknotic nuclei.

4.2.4.1 Quantification of BrdU incorporation index

Measurement of the 5-Bromo-2'-deoxyuridine (BrdU) incorporation index was possible after the cultured testis explants were treated with BrdU during the last 4h of the culture as based on Livera et al (2000). Three numbered slides per block (at least five serial sections apart) were deparaffinised, rehydrated and immunostained for BrdU incorporation using a sheep anti-BrdU polyclonal antibody (Fitzgerald Laboratories) and counterstained with haematoxylin, according to the protocol described in Chapter 2. The number of Sertoli cell nuclei that showed a clear positive immunoreaction to BrdU per 1000 counted, under a light microscope and a x63 objective, was converted to a percentage = the BrdU incorporation index. As BrdU was only incorporated in cells during proliferation, this value was also referred to as the proliferation index (PI). A mean from each block was calculated and at least seven blocks per treatment were analysed.

4.2.4.2 Quantification of apoptosis

Apoptotic cells were detected in situ by use of the TUNEL method as described by (Livera et al., 2000). There were no apoptotic cells seen in the in vitro experiment sections investigated, though the positive control sections (rat testis, d18) did confirm the protocol was successful. This finding is consistent with the absence of apoptosis in fetal rat testes over e19.5-e21.5 in vivo (Olaso and Habert, 2000).

4.2.5 Analysis of hormone secretions

Hormone measurements are one of the most sensitive endpoints for studying endocrine active compounds (O'Connor et al., 2002). To ensure that the explant culture system was viable, four separate treatments were added to the media to manipulate the hormone production level of the testis explants. The fetal testis has two major endocrine products:

testosterone and inhibin-B. Both of these hormones were targeted by the different treatments chosen and their effects compared to basal hormone production levels as measured in the media of untreated explants. More details on the doses and rationale behind these choices are provided in Chapter 2.

4.2.5.1 Measurement of testosterone production

The testosterone secreted into the media was measured by radioimmunoassay (RIA) as described in Chapter 2. No extraction step was performed as the only steroid that significantly cross-reacts with the testosterone antibody (17β-hydroxy-5α-androstane-3-one) is secreted in minute amounts by the fetal testis (Habert and Picon, 1984). Typically, a 1/1000 dilution of culture media was prepared for assay.

4.2.5.2 Measurement of inhibin-B production

The inhibin-B secreted into the media were measured in duplicate by an Enzyme Linked ImmunoSorbent Assay (ELISA) as described in Chapter 2. No pre-treatment of the media were necessary. Typically, a 1/20 dilution of culture media were used for assay.

4.2.6 Phthalate exposure

In studies where DBP is administered orally in vivo, the vast majority of DBP is reported to be converted to its metabolites, inlcuding MBP and MBP-glucuronide, which are then rapidly transferred to the embryonic tissues. As DBP metabolism is normally carried out by the liver, its metabolite MBP was utilised in the organ cultures to examine its effects on testis development (Saillenfait et al., 1998).

In their placental transfer study, Saillenfait et al. (1998) determined that just 0.12-0.15%, of the 500mg [14C]DBP/kg administered maternal dose, was recovered from the embryonic tissue. This equated to each fetus being exposed to just 0.6mg DBP/kg/day = 0.00216mM DBP/kg/day in a dam exposed to 500mgDBP/kg/day (1.8mM DBP/kg/day). It was considered useful to provide an equivalent dose of MBP in vitro to that received by the fetus following maternal treatment with 500mgDBP/kg in vivo. With these exposure levels in mind, the following experiments provided MBP or DBP at a maximum concentration of 1mM/ml ($1x10^{-3}$M), decreasing by tenfold each time to a minimum of 100pM/ml (10^{-10}M).

4.2.6.1 Phthalate effect on hormone stimulation

The details of the mechanism by which phthalates induce change in the development of the fetal testis is unclear, but appears to be associated with Leydig cell testosterone biosynthesis (Akingbemi et al., 2001). Initial experiments compared the level of testosterone and inhibin-B produced in vitro by explants treated with a range of doses of MBP, from 10^{-10}M to 10^{-3}M, against the levels produced by untreated, control, explants.

Further experiments were conducted to try to pinpoint the site of action of MBP. Explants were exposed to MBP together with hCG and 22-R-CHO which stimulate testosterone production by acting at different points in the steroidogenic cascade. By comparing whether the steroidogenesis stimulants exerted a reduced effect on testosterone production with MBP present, it could be pinpointed where in the steroidogenic pathway the DBP metabolite was having its inhibitory effect. These experiments were repeated with different concentrations of MBP to investigate any dose response effect.

4.3 Results

4.3.1 Optimisation of explant culture conditions

Studies were carried out to refine the conditions for the in vitro culture of fetal rat testis explants based on established in-house methods for the in vitro incubation of fetal human gonads, fetal rat Wolffian ducts and fetal rat prostate. Testes from fetuses at a range of embryonic ages were used, from e15.5 to e21.5, and several alternative culture conditions were investigated. One of the normal in vivo roles of the fetal testis is testosterone secretion, so levels of this hormone secreted into the culture media were measured as a means of quantifying the experimental outcomes. It was decided that increased testosterone production was an indication of better explant viability. Where possible, additional means of quantification were also employed, such as histological examinations or measurements of inhibin-B production.

4.3.1.1 Effect of incubation duration

Preliminary studies to measure the effect of incubation time were carried out for e17.5 and e19.5 fetal rat testis explants over 4-72h. The effects on the hourly rate of testosterone production and tissue histology were assessed. Fetal rat testis explants at e17.5 and e19.5 were incubated in vitro at 37°C for 4-72h without changing the media.

Differences in secreted testosterone were measured in media collected at the different timepoints. The greatest total testosterone secretion and hourly output was found in the media collected after 48h incubation for both ages (see Figures 4.1 and 4.2). It was concluded that an incubation length of 48h would be the focus for further studies.

The two experiments showed that the level of testosterone production differed with fetal age, this was followed up in further experiments (see section 4.3.1.4).

It is not clear from these studies how the level of testosterone decreased between 48-72h, as a linear effect of time on concentration was anticipated. It is considered that some of the testosterone must have undergone further metabolism after 48h.

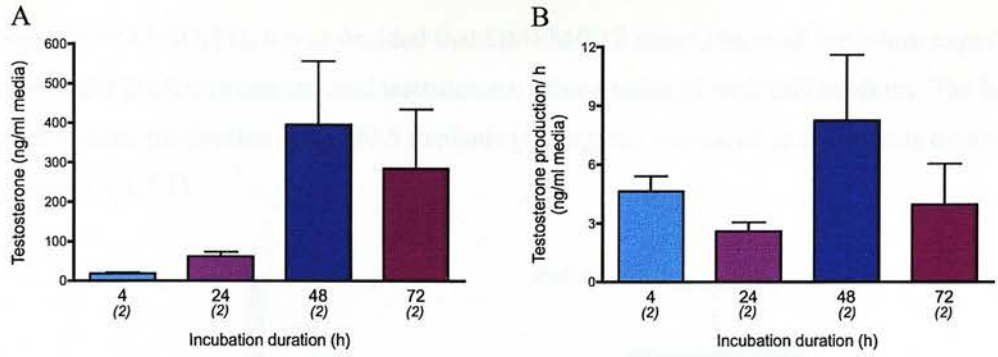

Figure 4.1: Testosterone production in vitro by e17.5 rat testis explants after culture for 4-72h at 37°C. Graph A shows the total amount of testosterone secreted over the incubation period and graph B shows average testosterone production per hour of incubation. (n values are shown in parentheses). Values are means (n=2) ± ranges.

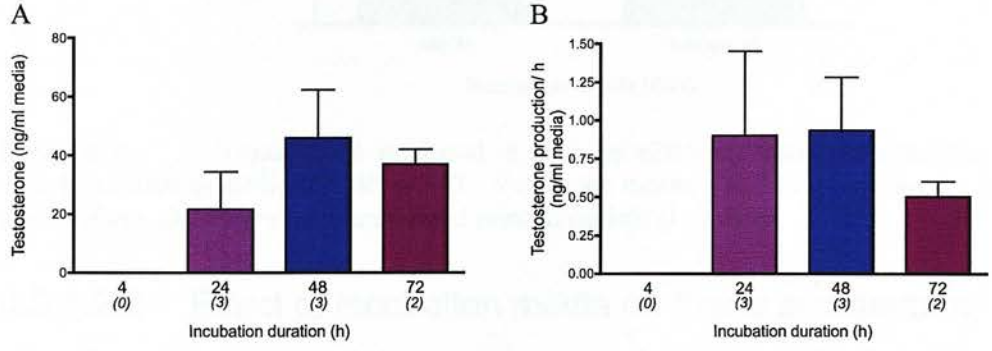

Figure 4.2: Testosterone production in vitro by e19.5 rat testis explants after culture for 24-72h at 37°C. Graph A shows the total amount of testosterone secreted over the incubation period and graph B shows average testosterone production per hour of incubation. (n values are shown in parentheses). Where n=2 values are means ± ranges but where n=3 Values are means ± S.E.M.

4.3.1.2 Effect of incubation media

The two in-house protocols for in vitro tissue culture used different culture media for explant incubation at 37°C. The effects of the different basic media on the fetal rat testis explants were assessed. The levels of testosterone secretion and quality of tissue histology were compared. The two basic media used were Alpha Minimum Essential Media (AMEM) and a 1:1 mixture of Dulbecco's Minimum Essential Media with F12 (DMEM/F12), both supplied by Gibco™. Identical anti-microbial products were added to all media. Following termination of the incubation, the residual media were collected per well and its testosterone level measured.

4.3.1.2.1 Effect of incubation media on testosterone production

A noticeable difference in the level of testosterone production between the media was observed after 48h incubation (Figure 4.3). Though this difference was not statistically

significant (P=0.081), it was decided that DMEM/F12 should be used for future experiments due to the greater mean secreted testosterone value produced with this medium. The low testosterone production with e20.5 explants (<50ng/ml) compared to a previous experiment (section 4.3.1.1).

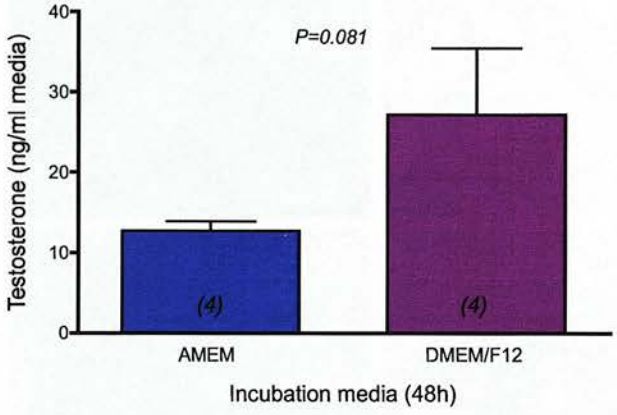

Figure 4.3: Testosterone produced in vitro by e20.5 rat testis explants after culture with two different media for 48h at 37°C. Values are means ± S.E.M. (n values are shown in parentheses). Data were log transformed prior to statistical analysis.

4.3.1.2.2 Effect of incubation media on tissue architecture

Following incubation, the explants were Bouin's fixed then processed into paraffin blocks and sectioned. Prior to analysis, testis sections were immunostained for two cell-specific markers (Figure 4.4). Low magnification examination of the sections reviewed the steroidogenic potential of the explants following immunostaining for the enzyme 3β-HSD. This protein is unique to the Leydig cells in the fetal testis and is critical to the biosynthesis of testosterone. All cultured explants showed a decrease in the intensity of 3β-HSD staining compared to uncultured control sections. At 24h, the 3β-HSD staining intensity was comparable between the media, but by 48h, the AMEM cultured tissue showed markedly less intense staining. Explant architecture was examined using immunostaining for the cytoskeletal protein Smooth Muscle Actin (SMA). This protein is unique to the peritubular myoid cells in the fetal testis and contributes to the physical support of the developing seminiferous cords. All cultured explants showed a change in SMA staining intensity with time and a markedly changed pattern of expression at 48h compared to 0h control sections. Review of the counterstained regions of the sections revealed pyknotic cell nuclei in all cultured explants though none in the 0h controls. For both media, the most vulnerable cell types were the intratubular gonocytes and interstitial Leydig cells. However, those explants

cultured in DMEM/F12 appeared to have less frequent pyknotic cell nuclei though this was not quantified and was considered an artefact of the in vitro procedure (Figure 4.4).

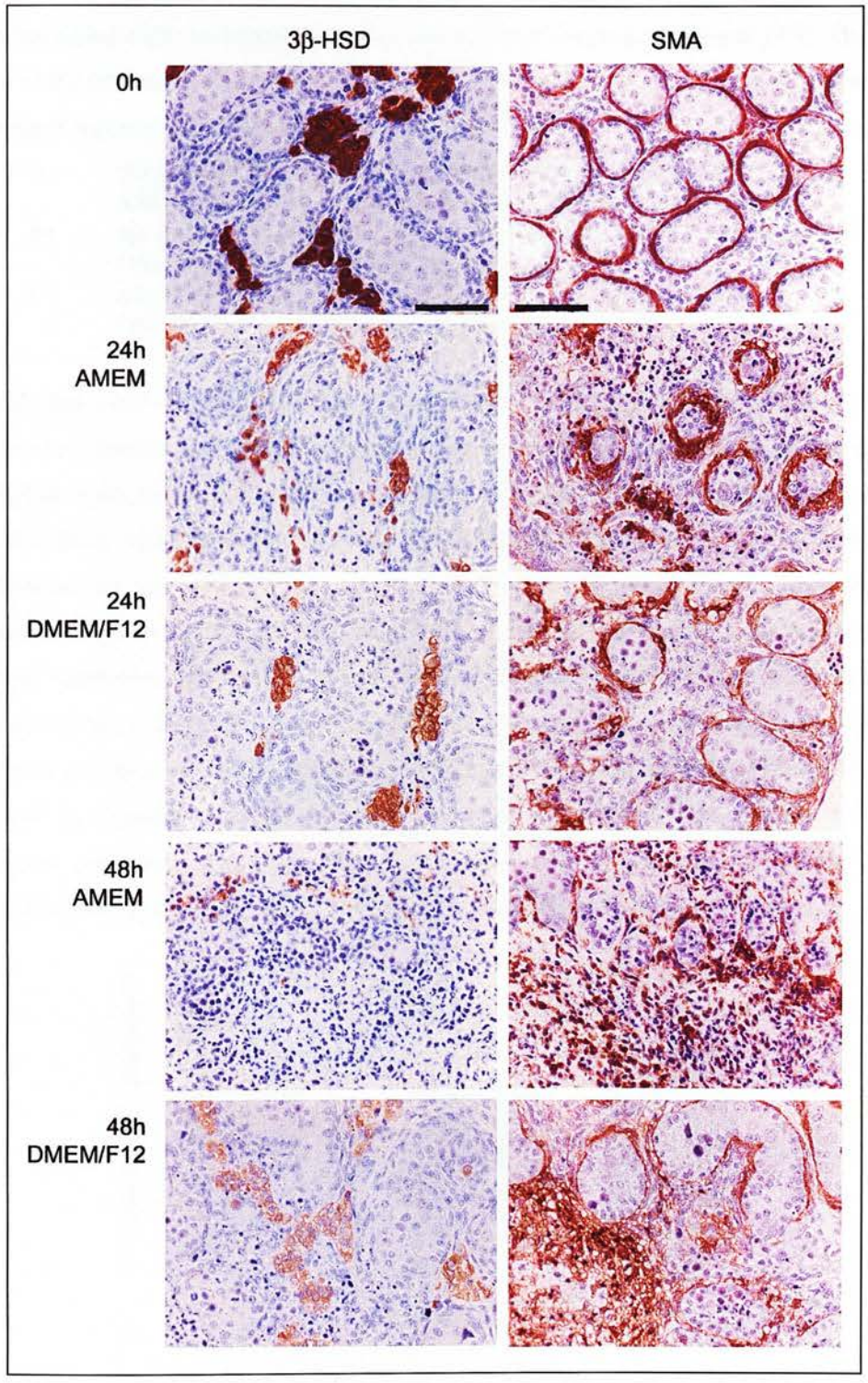

Figure 4.4: Histological examination of e20.5 fetal rat testis explants after culture for 0, 24 or 48h in AMEM or DMEM/F12 media. Sections were immunostained for the Leydig cell marker 3β-HSD or the peritubular cell marker SMA. Scale bars denote 100μm.

4.3.1.3 Effect of media supplements

The two established in-house protocols for in vitro tissue culture not only used different media but added different supplements too, prior to fetal tissue incubation at 37°C. The effects of the different supplements on the fetal rat testis explants were assessed. Three supplement regimes were tested (details in section 4.2.2).

- A: the original **AMEM** regime for fetal human gonad culture
  AMEM media + L-Glutamine + sodium pyruvate + ITS + BSA
- B: the **original DMEM/F12** regime for the rat tissue culture experiments
  DMEM/F12 media + transferrin + insulin
- C: a hybrid of the 2 regimes with a **modified DMEM/F12** regime
  DMEM/F12 + L-Glutamine + sodium pyruvate + ITS + BSA

These studies were run in parallel with the media trial (section 4.3.1.2). The levels of testosterone secretion and Sertoli cell proliferation rates were compared between regimes. The highest mean level of testosterone after 48h incubation (28.2ng/ml media) was measured in media from explants incubated with the modified DMEM/F12 regime (Figure 4.5). Proliferation of the explants towards the end of the experiment was measured by immunostaining the explant sections for BrdU, which had been added to the media for the last 4h of incubation. The Sertoli cell proliferation index was calculated from sections double immunostained for BrdU and WT-1 (Figure 4.7). There was no obvious difference between the media regimes on the proliferation rate though the greatest mean PI% (3.4%) was measured in explants incubated with the modified DMEM regime (Figure 4.6). The differences seen were not statistically significant but it was decided that future culture experiments would use the modified DMEM/F12 media and supplement regime.

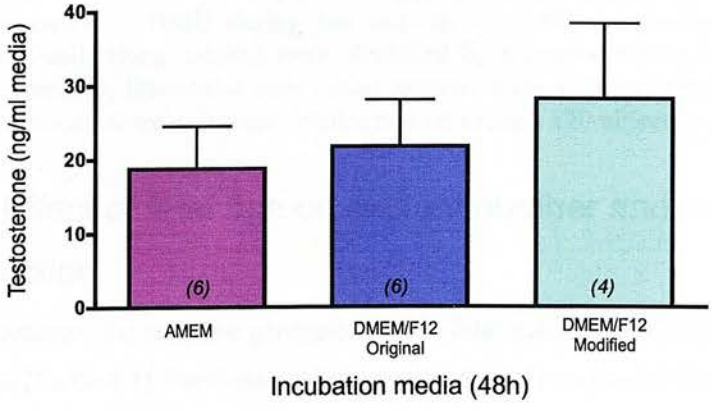

Figure 4.5: Testosterone production in vitro by e20.5 rat testis explants after culture with three different media regimes for 48h at 37°C. Values are means ± S.E.M. (n values are shown in parentheses). There was no significant effect of media regime (P=0.67).

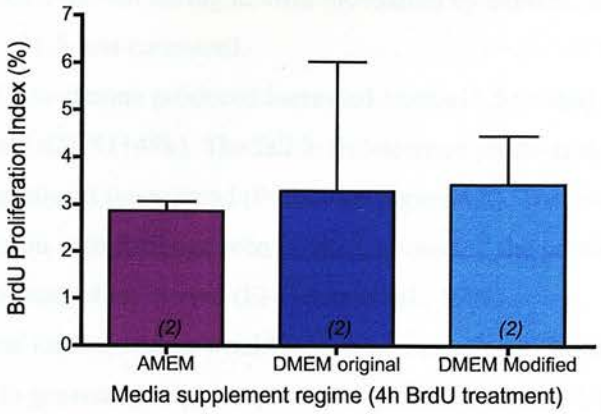

Figure 4.6: Proliferation Index (%) of Sertoli cells in e20.5 fetal rat testes following 4h in vitro exposure to BrdU at the end of a 48h incubation. Values are means ± range. (n values are shown in parentheses).

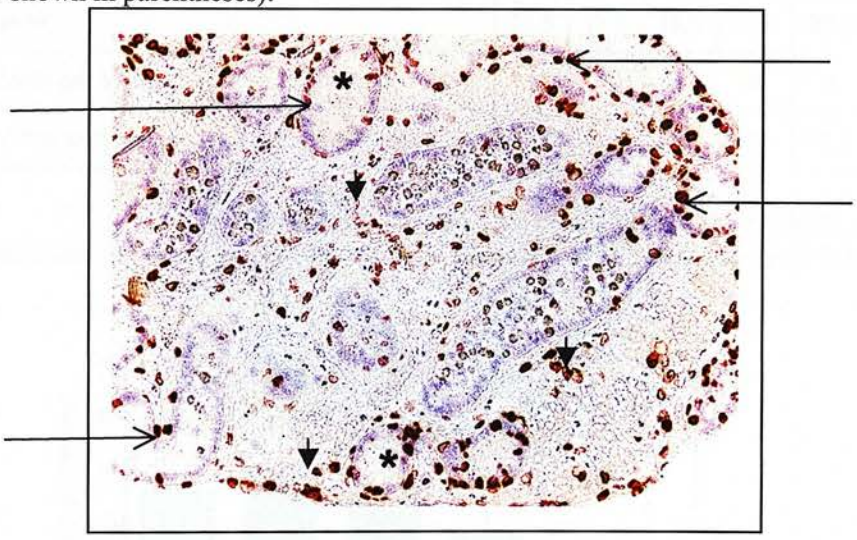

Figure 4.7: BrdU and WT-1 double-immunostained section of e20.5 fetal rat testis after in vitro exposure to BrdU during the last 4h of a 48h incubation. Proliferating intratubular Sertoli cells (long arrows) were identified by immunostaining for WT-1 (blue) as well as BrdU (brown). Interstitial cells (short arrows) were also proliferating at this age but gonocytes (asterixes) were quiescent. Photographed using a x20 objective.

4.3.1.4 Effect of fetal age on explant number and testosterone production

The number of explants that could be generated from a fetal testis increased as the testis enlarged with age (Table 4.1). Previous studies (section 4.3.1.1) suggested that the age of the testis donor, at the time of explant preparation, influenced the level of testosterone produced by the explant in culture, so this was investigated. It was not possible to reproducibly retrieve the male gonad from fetuses younger than e15.5 and it has been reported that testosterone production is not detected prior to e15.5 (Rouiller-Fabre et al., 2003). The level of

testosterone produced over 48h during in vitro incubation by explants retrieved from fetuses aged from e15.5 to e21.5 was compared.

The mean levels of testosterone produced increased from e15.5 (+48h) to e17.5 (+48h) and then fell steadily until e21.5 (+48h). The fall in testosterone production after e17.5 (+48h) showed a highly significant linear trend (P=0.004) (Figure 4.8). The rise and fall in testosterone production with fetal age seen in vitro, mimicked the previously reported pattern of fetal testosterone production in vivo (El-Gehani et al., 1998).

It was concluded that further studies would be carried out using e19.5 rat fetal testes as these were large enough to generate 6 explants per testis but still showed a high level of testosterone production. This would mean more replicates could be generated per testis so fewer animals would be needed to achieve the same sample size per experiment.

Fetal age (e)	15.5	17.5	18.5	19.5	20.5	21.5
No. explants per testis	1	2	4	6	8	8+
Mean in vitro testosterone production (ng/ml medium)	36.3	41.4	37.5	27.7	17.0	5.25

Table 4.1 A summary of the effect of fetal age on the number of explants generated per fetal testis and the mean level of testosterone produced over 48h in vitro (see Figure 4.8).

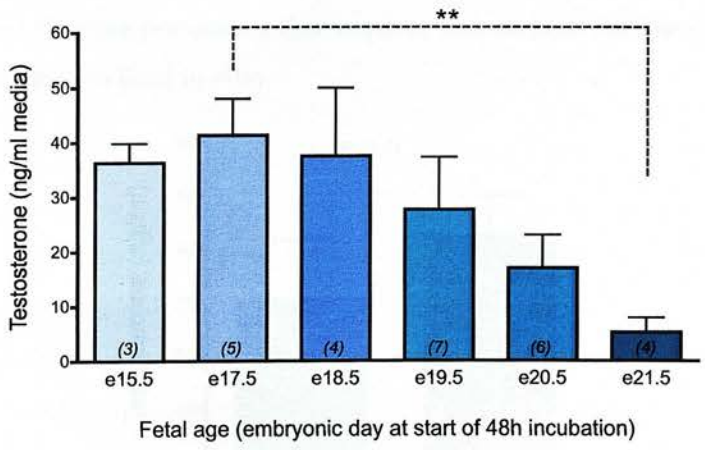

Figure 4.8: Testosterone production in vitro by rat testis explants from fetuses of various ages after incubation for 48h at 37°C. Values are means ± S.E.M. (n values are shown in parentheses). Data were log transformed prior to statistical analysis. Testosterone levels peaked with the e17.5 explants then fell steadily with increasing age (linear trend analysis of decrease P=0.004**).

4.3.1.5 Effect of dissection plane on explant function

The fetal testis has an organised architecture that is populated by cells proliferating in situ (e.g. Sertoli cells) and by cells that initially migrate in from surrounding tissue, from the

mesonephros and/or coelemic epithelium (e.g. peritubular myoid cells, Leydig cells) or the yolk sac (i.e. gonocytes) (Karl and Capel, 1998; Olaso and Habert, 2000; Ross et al., 2003). Explants were generated for in vitro culture by slicing whole fetal testes into even sized, smaller pieces. It was proposed that slicing of the ovoid testis would unavoidably disrupt any on-going cell migration from the coelomic epithelium or the dispersal of cells from the rete after migrating from the mesonephros. Regulating the plane of dissection would minimise any disruption of residual migration, or at least ensure it was consistent between explants. Equally the uneven distribution of the different cell types within each explant, associated with migration or cell number, was minimised using this approach.

The benefit of this precaution was assessed by comparing testosterone output between samples generated either with careful orientation of the whole testis and specific planes of dissection or by slicing the whole testis with random regard for the organ's orientation. Following incubation for 48h at 37°C, the media were collected and testosterone levels were compared (Figure 4.9). It was not possible to track the orientation of explants after they were generated, thus prohibiting any quantitative histological comparisons.

There was no significant effect of dissection plane on the level of testosterone production (P=0.41) but it was decided that future culture experiments would employ the specific orientation method. This method would generate explants that were comparable in testicular architecture and the extra preparation time required had not been detrimental to the explants' testosterone production level in vitro.

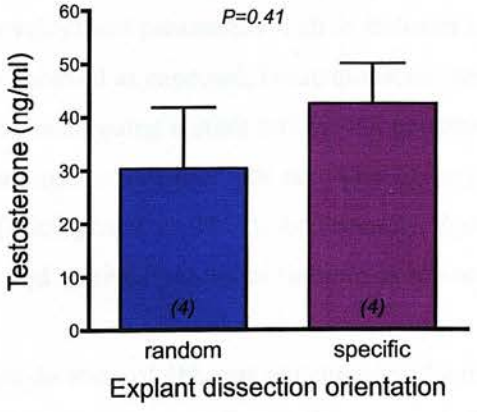

Figure 4.9: Levels of testosterone (ng/ml media) produced in vitro by e19.5 rat testis explants with different explant dissection orientation after culture for 48h. Values are means ± S.E.M. (n values are shown in parentheses). Data analysis showed no significant difference between the two groups (P=0.41).

4.3.1.6 Optimisation procedures: conclusion

Optimisation of culture condition has been shown to be critical to ensure a useful in vitro representation of in vivo development (Richards, 1999). The in vitro organotypic system developed in these studies was assessed using three main criteria:

- maintenance of tissue architecture and protein expression (Figure 4.5)
- measurement of hormone production (Figure 4.6)
- analysis of the somatic cell proliferation rate (Figure 4.7)

Regime C, as described in section 4.3.1.3, was the most successful set-up based on these criteria so was used to incubate rat e19.5 testis explants for 48h during subsequent experiments.

The level of testosterone produced by the fetal testis explants varied depending on the fetal age at which they were collected. This trend was consistent with the pattern of testicular testosterone content seen in whole testes, confirming that the in vitro explant culture system did mimic in vivo testosterone production (El-Gehani et al., 1998). The number of explants that could be generated per testis was proportionate to the age and hence size of the testis (Livera et al., 2004). The most explants were produced from the oldest testes (e21.5). This benefit (more explants per testis = more experiments per animal = reduced animal usage) was carefully weighed against the fall in testosterone production with age. The optimal situation decided upon was to collect testes on e19.5, when testosterone production had peaked but not plummeted and 2-3 times more explants could be generated per testis than at e17.5. This would enable more replicates per experiment to be generated to highlight any effect of treatment on development parameters such as testosterone production apart from in vitro effects. Gonocytes behaved as expected, being quiescent across the developmental period used (e19.5 +48h), undergoing neither mitosis nor apoptosis. In this organotypic culture system, the absent mitotic and apoptotic activities of the gonocytes were similar to those observed in vivo (Boulogne et al., 1999). Additionally, Sertoli cells and Leydig cells underwent proliferation and secreted hormones (inhibin-B and testosterone, respectively) as per in vivo.

The optimised incubation duration of 48h was not considered a limitation of the technique as this timeframe enabled chronic exposure effects to be assessed. There were certain artefacts induced by this experimental approach that could not be removed despite method optimisation. For example, most explants showed poor tissue morphology in their centre after 48h incubation. The necrotic cells were recognisable microscopically by their darkly counterstained pyknotic nuclei. Ideally, all necrotic tissue would have been discarded from further analysis but due to the consistent appearance of this artefact in the centre of cultured

explants, necrotic tissue was noted but investigative focus was placed on the viable peripheral tissue per sample.

4.3.2 Manipulation of hormone secretion in vitro

The refined in vitro model with fetal rat testis explants was exposed to four agents, three of which are known to affect steroidogenesis, to investigate whether hormone production could be manipulated. hCG and 22-R-CHO were selected to stimulate testosterone production, at different points in the steroidogenic cascade, and to assess Leydig cell vitality. Ketoconazole (KTZ) was chosen as an inhibitor of testosterone production. FSH treatment was intended to stimulate inhibin-B production and assess Sertoli cell vitality, which might have an indirect effect on steroidogenesis. Further details on these compounds and the rationale behind their selection are provided in Chapter 2.

4.3.2.1 Manipulation of testosterone secretion

Fetal rat testis explants at e19.5 were incubated in vitro at 37°C for 48h. Media were prepared with or without the four treatments described above and were collected after termination of the incubation. Analysis of media for testosterone from preliminary studies (Figure 4.10 A, B and C), illustrates the range of levels for basal testosterone production and the magnitude of response, if any, the treatments induced. In all experiments shown, the 22-R-CHO and hCG treatments induced increased testosterone production, as anticipated. In contrast, the addition of FSH or KTZ had inconsistent effects on testosterone levels compared to the control samples. Because of the variability in results, these studies were repeated in several experiments.

The media collected from multiple studies (repeated 4-7 times) were assayed together prior to overall analysis (Figure 4.11). Data were log transformed prior to statistical analysis. For testosterone, ANOVA showed $P<0.0001$ with high significance ($P<0.01$) for both 22-R-CHO and hCG compared to basal control. The maximal increase in hCG induced testosterone production (mean = 177ng/ml) under these conditions, was almost 6 fold greater than basal levels (mean = 31ng/ml) but a 13 fold increase was induced by the addition of 22-R-CHO (mean = 396ng/ml).

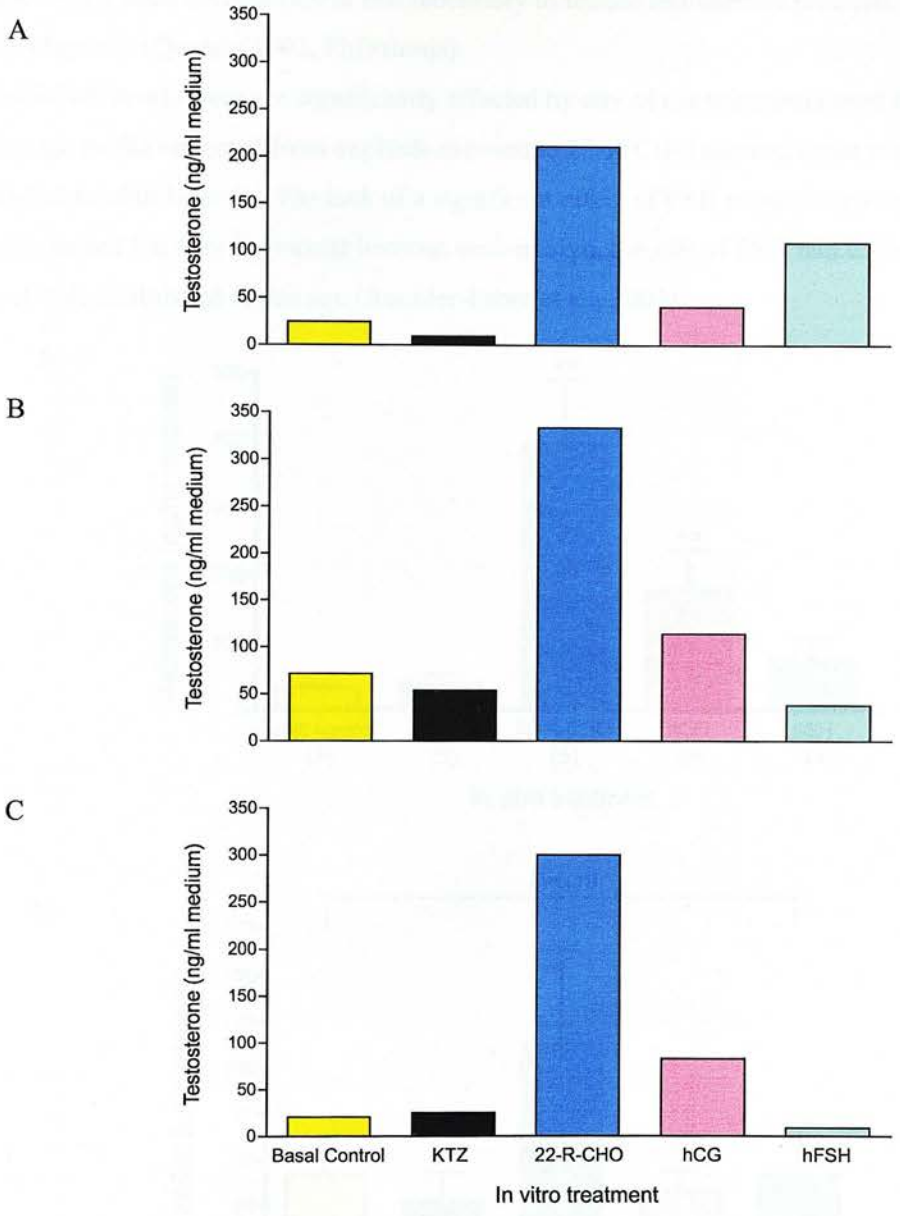

Figure 4.10: Testosterone production in vitro by e19.5 rat testis explants after culture for 48h with different culture media additions. Each graph (A, B, C) represents data from a different single experiment. Individual experiments were from a single litter. All treatment groups had the same amount of tissue, as determined by number of explants and their size.

In contrast, there was no significant effect of FSH or KTZ on testosterone production compared to untreated samples ($P>0.05$) (Figure 4.11 A). No significant effect of FSH was expected on testosterone production, so these results were consistent with expectations. However, the lack of adverse effect on testosterone level by KTZ was unexpected. It must be that the concentration used was ineffective under these culture conditions, though it was

previously used successfully in this laboratory to reduce testosterone production by adult rat Leydig cells (Qureshi 1992, PhD thesis).

Inhibin-B levels were not significantly affected by any of the treatments used (Figure 4.11 B) though media collected from explants exposed to 22-R-CHO showed some evidence of higher inhibin-B levels. The lack of a significant effect of FSH on inhibin-B secretion was unexpected but may be normal because even in vivo, the role of FSH and its production is not well established at this age (Rouiller-Fabre et al., 2003).

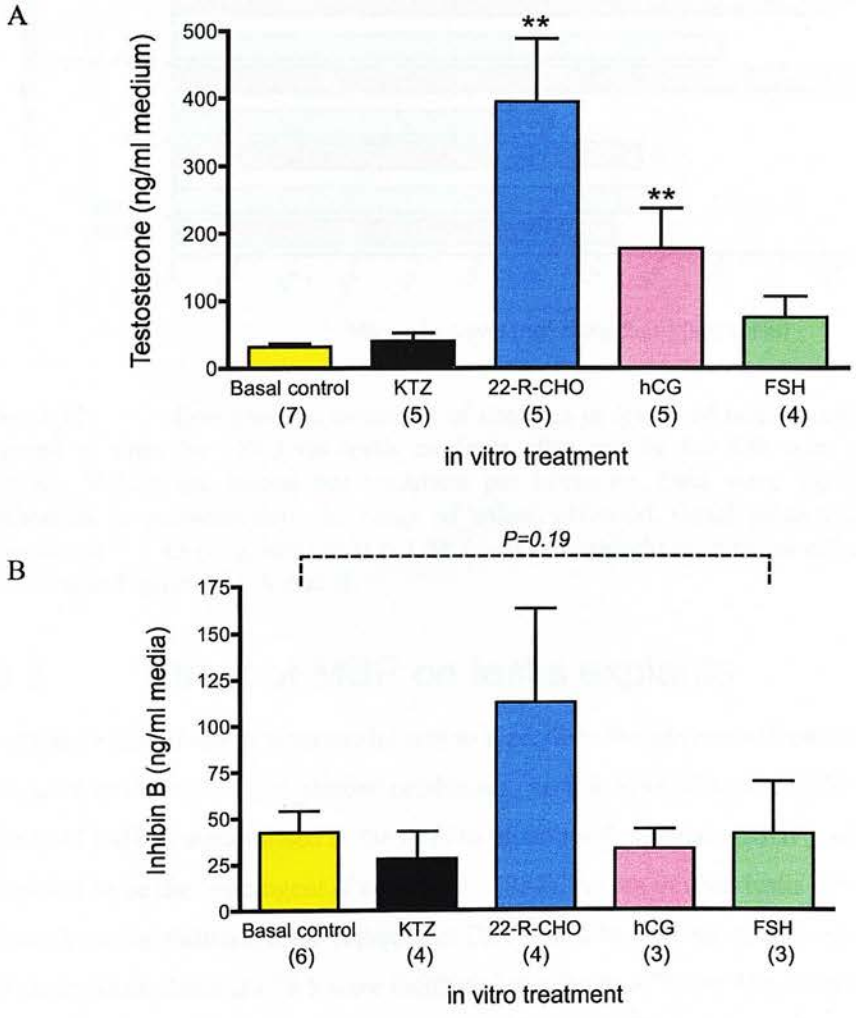

Figure 4.11: Production of testosterone (A) and inhibin-B (B) in vitro by e19.5 rat testis explants after culture for 48h with different media additions. Values are means ± S.E.M. (*n* values are shown in parentheses). For testosterone data, values were log transformed prior to analysis to normalise the wide range of values obtained. ANOVA showed P<0.0001 with high significance (** P<0.01) for both 22-R-CHO and hCG compared to control. ANOVA analysis showed no effect of treatment on inhibin B levels (P=0.19) compared to untreated control samples.

A comparative summary of the two hormones and the mean effect of each in vitro treatment is presented in Figure 4.12 which plots the relative increases in overall hormone production per treatment. It was possible neither to reduce the level of testosterone production using these treatments nor to significantly affect inhibin-B levels.

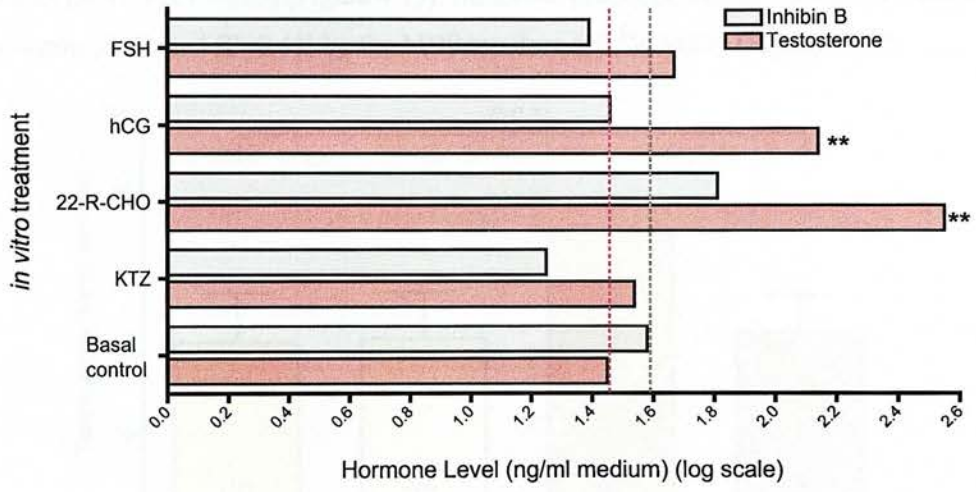

Figure 4.12: Comparative summary of changes in levels of testosterone and inhibin-B produced in vitro by e19.5 rat testis explants after culture for 48h with different media additions. Values are means per treatment per hormone. Data were log transformed for presentation to accommodate the range of values obtained. Basal mean values are plotted (testosterone = 1.45 (---), inhibin B = 1.58 (---)) to highlight significant effects of treatment as detailed in Figure 4.11 A and B.

4.3.3 Effect of MBP on testis explants

The ultimate aim of the in vitro model was to reproduce the adverse effects of DBP on fetal testes, such as decreased testosterone production, seen in vivo (Chapter 3). In rats, the vast majority of DBP is metabolised in the liver to monobutyl phthalate (MBP), which is understood to be the toxic agent (Foster et al., 1983). As the in vitro testis model cannot perform liver metabolism, MBP rather than DBP was added to the culture media.

Fetal rat testis explants at e19.5 were incubated in vitro at 37°C for 48h, with or without MBP at concentrations varying from 10^{-3}M (1mM) to 10^{-10}M (100pM). Media collected after 48h incubation was assayed for testosterone and inhibin-B and explants were Bouin's fixed, sectioned and immunostained as required.

4.3.3.1 Effect of MBP on testis explant hormone production

Testosterone levels measured in media from preliminary studies showed wide variability between the experiments (Figure 4.10) so these cultures were repeated 5-17 times. The

samples from multiple studies were assayed together prior to overall analysis (Figure 4.13). Data were log transformed prior to statistical analysis.

Analysis of data from repeated studies, showed that basal testosterone production by e19.5 explants was unaffected by MBP at any of the concentrations used, compared to untreated explants (ANOVA P=0.23) (Figure 4.13). Inhibin-B production by e19.5 explants was not significantly affected (P=0.41) by the MBP top dose (10^{-3}M MBP) (Figure 4.14).

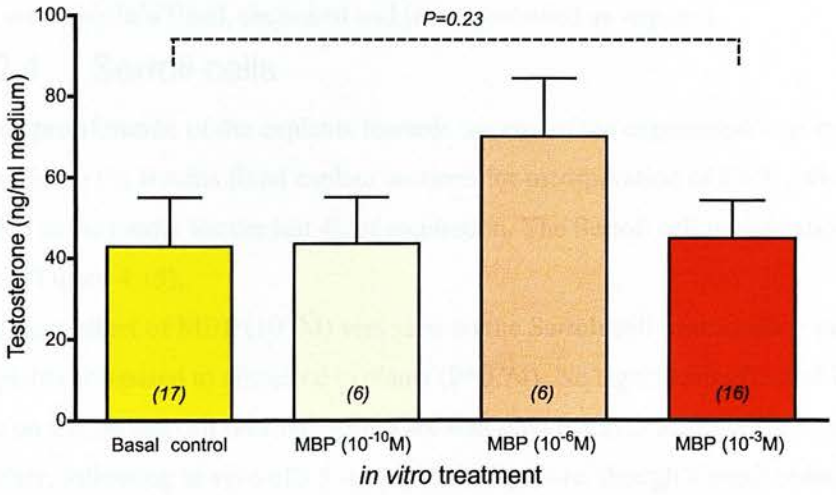

Figure 4.13: Testosterone production in vitro by e19.5 rat testis explants after culture with different MBP concentrations for 48h at 37^{0}C. Values are means ± S.E.M. (n values are shown in parentheses). ANOVA analysis showed no effect of dose level (P=0.23) compared to untreated control samples.

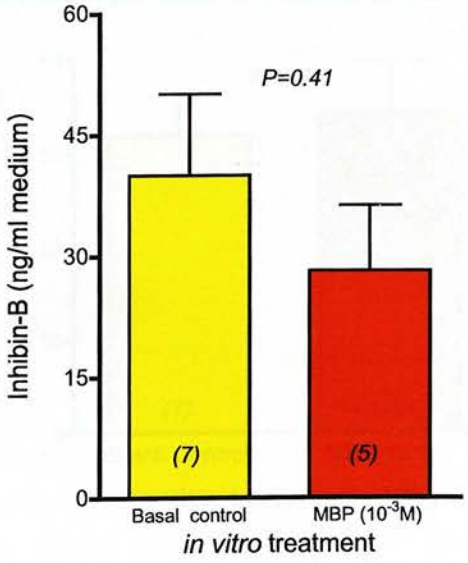

Figure 4.14: Inhibin-B production in vitro by e19.5 rat testis explants after culture with or without MBP(10^{-3}M) for 48h at 37^{0}C. Values are means ± S.E.M. (n values are shown in parentheses). T-test analysis showed no effect of MBP (P=0.41) on inhibin production compared to basal samples.

4.3.3.2 Effect of MBP on testis explant histology

The ultimate aim of the in vitro model was to reproduce the adverse effects of DBP on fetal testes, seen in vivo (Chapter 3). Fetal testes at e21.5 exposed to DBP in utero over e13.5-e20.5, showed reproducible changes in certain aspects of their cellular architecture (section 3.3). The same parameters were assessed in fetal testes, explanted at e19.5 then incubated in vitro with or without the DBP metabolite MBP for 48h. Following in vitro incubation, explants were Bouin's fixed, sectioned and immunostained as required.

4.3.3.2.1 Sertoli cells

Sertoli cell proliferation of the explants towards the end of the experiment was measured by immunostaining the Bouins fixed explant sections for incorporation of BrdU, which had been added to the media for the last 4h of incubation. The Sertoli cell proliferation index was calculated (Figure 4.15).

No significant effect of MBP (10^{-3}M) was seen on the Sertoli cell proliferation index of fetal testis explants compared to untreated explants (P=0.74). No significant effect of DBP exposure on the Sertoli cell proliferation index was seen in e21.5 testes with the in vivo model either, following in vivo e13.5 -e20.5 DBP exposure, though a small reduction in Sertoli cell number was measured (section 3.3.1.1). Sertoli cell number was not measured in fetal testis explants after incubation in vitro due to the necrosis mentioned earlier (section 4.1.1.6). BrdU uptake was evident but the PI% was not measured for extratubular cell types and gonocytes were quiescent, as per the in vivo studies (Figure 4.16).

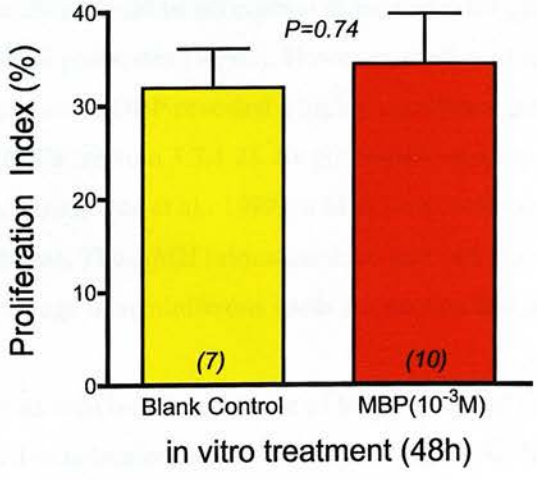

Figure 4.15: Proliferation Index (%) of Sertoli cells in e19.5 rat testis explants during the last 4h of 48h in vitro incubation with or without MBP(10^{-3}M), at 37°C. Values are means ± S.E.M. (n values are shown in parentheses). There was no significant effect of MBP on the Sertoli cell PI%.

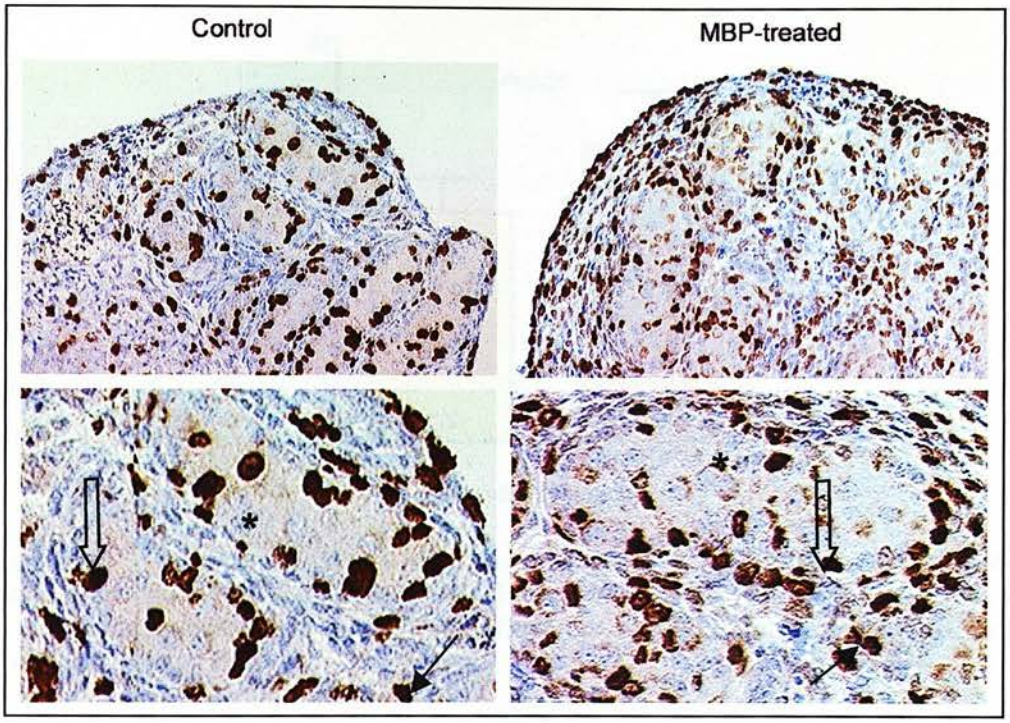

Figure 4.16: BrdU immunostained sections of e19.5 fetal rat testis explants after culture for 48h, with or without MBP(10^{-3}M). BrdU was administered 4h prior to incubation termination and was incorporated by the proliferating cells including intratubular Sertoli cells (wide arrows) and interstitial cells (narrow arrows) but gonocytes appeared quiescent (asterixes). The top panels show cross sections through testis explants photographed with a x20 objective, with focal areas enlarged below.

4.3.3.2.2 Gonocytes

Normal untreated/ uncultured fetal testes contain mononucleated gonocytes, with rare and infrequent multinucleated gonocytes (MNG). However, studies of testis architecture following in utero exposure to DBP revealed a highly significant treatment related increase in the incidence of MNG's (section 3.3.1.2). As gonocytes were not typically mitotic during the fetal age examined (Boulogne et al., 1999), a MNG was defined as having more than one nucleus per cell membrane. The AMH immunostained sections from cultured explants were analysed and the percentage of seminiferous cords per section that contained at least one MNG was recorded.

No significant effect was seen on the incidence of MNG's in fetal testis explants treated with MBP(10^{-3}M) compared to untreated explants (P=0.31) (Figure 4.17). The unexpectedly high incidence of MNG's, seen with the control explants, was considered abnormal and is presumed to be a consequence of the in vitro culture, and may perhaps have masked any significance of the greater mean incidence of MNG's seen in the fetal testis explants treated with MBP(10^{-3}M) compared to untreated explants (Figure 4.18).

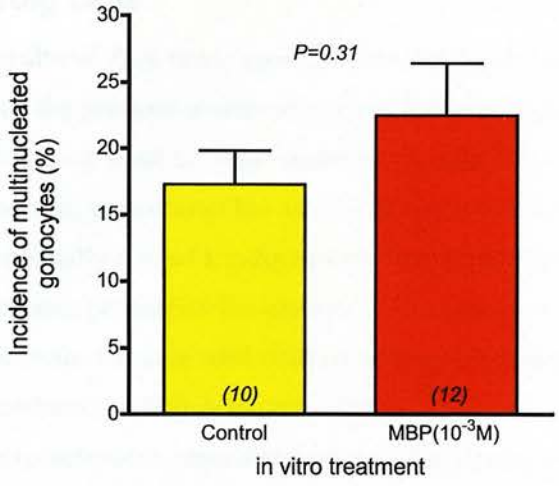

Figure 4.17: Incidence (%) of multinucleated gonocytes in e19.5 rat testis explants after in vitro culture with or without MBP(10^{-3}M), for 48h at 37^{0}C. Values are means ± S.E.M. (n values are shown in parentheses). There was no significant treatment effect and t-test analysis showed P=0.31.

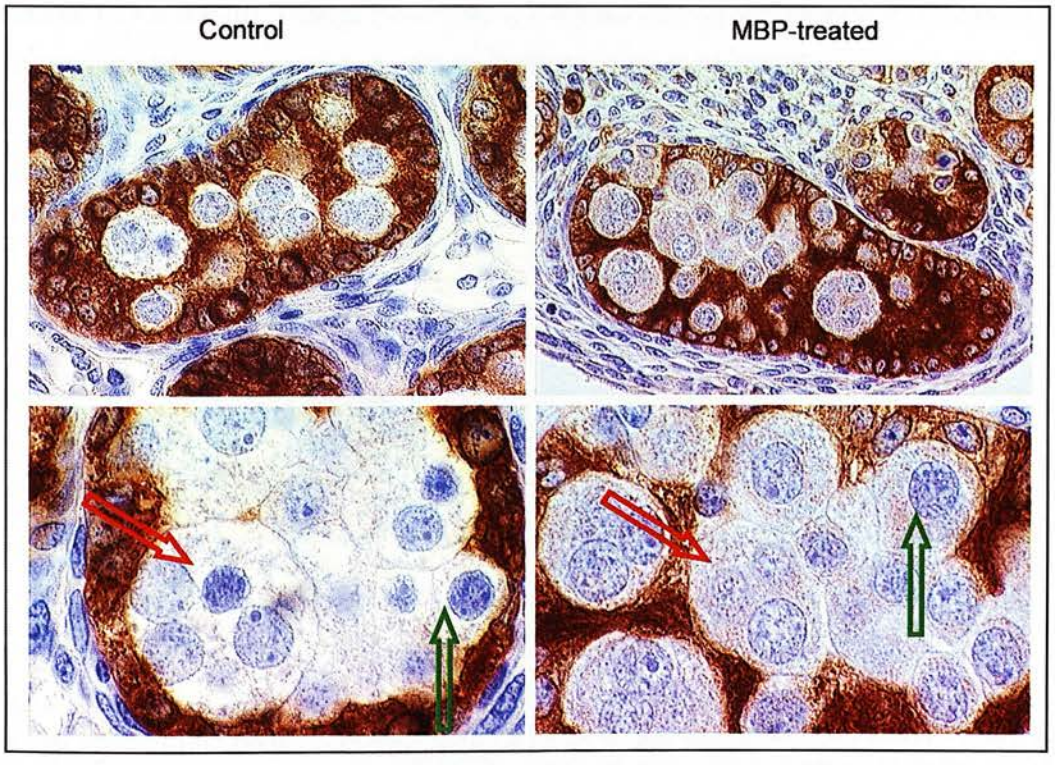

Figure 4.18: Examples of mononucleated (green arrows) and multinucleated (red arrows) gonocytes in e19.5 rat testis explants after in vitro culture with or without MBP(10^{-3}M), for 48h at 37^{0}C. Sections were immunostained for AMH (Sertoli cell cytoplasm in brown) and counterstained with haematoxylin (blue). The montage shows cross sections through explants photographed with a x40 objective in the top panels and re-photographed with a x100 objective in the lower panels.

4.3.3.2.3 Leydig cells

Normal untreated/ uncultured fetal testes aged between e19.5-e21.5, contain small clusters of Leydig cells. However, the previous studies of testis architecture following in utero exposure to DBP recorded a shift from small to larger clusters of Leydig cells with treatment (section 3.3.1.3). Without treatment, each cluster has an area of less than 5% of the total Leydig cell area per section. Occasionally normal Leydig cell clusters would represent between 5-15% of the total Leydig cell area per section but clusters >15% were only seen in testis sections from DBP-exposed animals. Clusters were defined by these percentage area categories as either small (<5%), medium (5-15%) or large (>15%).

Sections of cultured explants were immunostained with the Leydig cell marker 3β-HSD and the number of Leydig cell clusters per category was analysed. Three sections per sample were analysed and the mean values per sample compared between treatments. There was a trend away from smaller clusters towards an increase in the incidence of medium and large clusters seen in explants exposed in vitro to MBP (10^{-3}M) compared to untreated explants (Figures 4.19 and 4.20). Though the change was not statistically significant for any cluster category, this trend was consistent with the findings of the in vivo study.

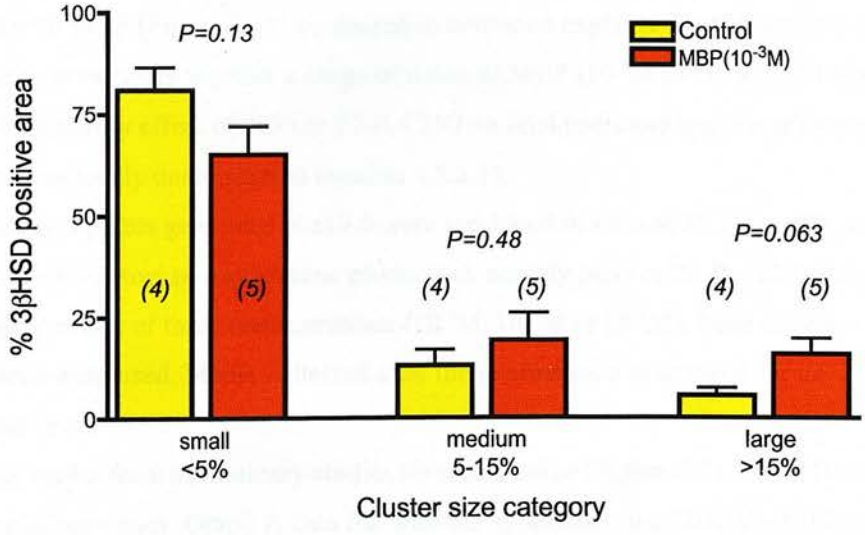

Figure 4.19: Leydig cell cluster size index (%) of total 3β-HSD immunopositive stained area in e19.5 rat testis explants after in vitro culture with or without MBP(10^{-3}M), for 48h at 37°C. Values are means ± S.E.M. (n values are shown in parentheses). MBP(10^{-3}M) treated explants showed a trend towards an increase in larger cluster sizes though t-test analysis showed no significant difference in any of the cluster categories between control and MBP exposed explants.

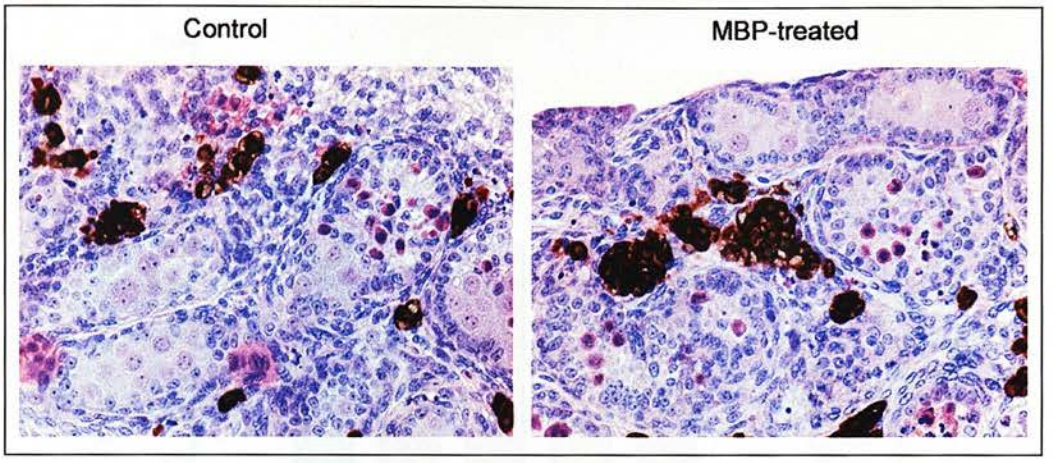

Figure 4.20: 3β-HSD immunostained sections of e19.5 rat testis explants after in vitro culture with or without MBP(10^{-3}M), for 48h at 37^{0}C. Note the range of Leydig cell cluster sizes, exaggerated after MBP-treatment, compared to the control image. The panels show cross sections through testis explants photographed with a x40 objective.

4.3.3.3 Effect of MBP on testosterone production by fetal testis explants stimulated by 22-R-CHO or hCG

There was no significant change in the level of testosterone produced by testis explants incubated with MBP (Figure 4.13) compared to untreated explants. Further studies were carried out to investigate whether a range of doses of MBP (10^{-9}M to 10^{-3}M MBP) could blunt the stimulatory effect of hCG or 22-R-CHO on fetal testis explant testosterone production previously demonstrated (section 4.3.2.1).

Fetal rat testis explants generated at e19.5 were incubated in vitro at 37°C for 48h, with or without two stimulators of testosterone production, namely hCG or 22-R-CHO and with or without MBP at one of three concentrations (10^{-9}M, 10^{-6}M or 10^{-3}M). Nine different combinations were used. Media collected after the incubation was assayed for its testosterone level.

Analysis of media from preliminary studies for testosterone (Figure 4.21 A and B) saw a wide range of responses. Graph A data fits with the hypothesis that MBP could blunt stimulated testosterone production in a dose related manner but this result was not supported by the repeat experiment in graph B. In both the experiments shown, hCG combined with MBP (10^{-3}M) saw blunted testosterone production compared to hCG alone. No reduction in the 22-R-CHO stimulated testosterone level was induced consistently by any MBP concentration.

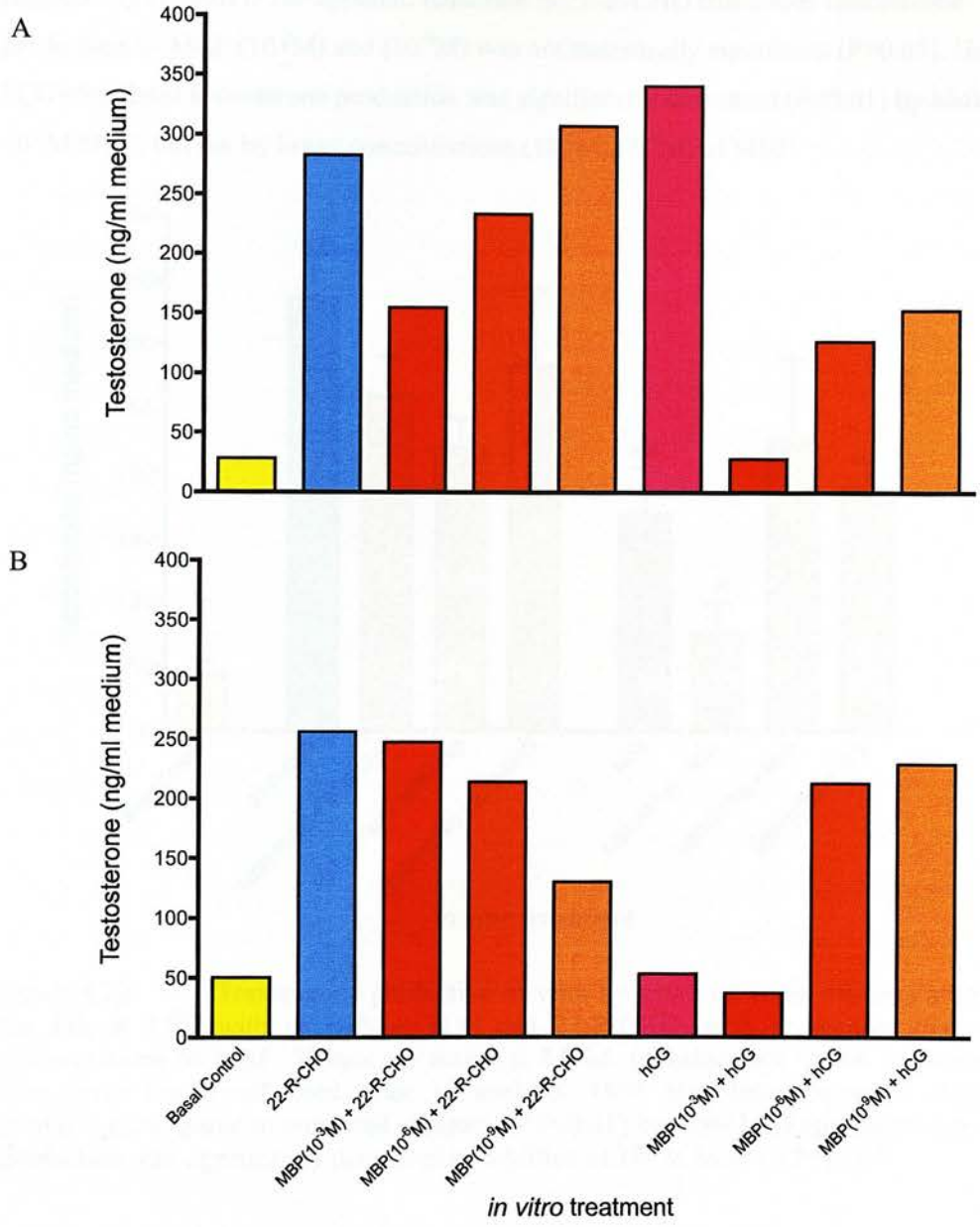

Figure 4.21: Testosterone production in vitro by e19.5 rat testis explants after culture for 48h at 37^0C with or without hCG and 22-R-CHO, with or without one of three concentrations of MBP. Each graph (A and B) represents data from a different single preliminary experiment.

Because of the variability in results, these studies were repeated (Figure 4.22). The samples from multiple studies were assayed together prior to overall analysis and data were log transformed prior to statistical analysis. Analysis of media for testosterone from multiple studies, showed that both stimulants increased testosterone production compared to untreated explants achieving 7.8 and 3.7 mean fold increases induced by 22-R-CHO and hCG

respectively (P<0.01). The apparent reduction in 22-R-CHO stimulated testosterone production by MBP (10^{-3}M) and (10^{-6}M) was not statistically significant (P>0.05). However, hCG stimulated testosterone production was significantly decreased (P<0.01) by addition of 10^{-3}M MBP, but not by lower concentrations (10^{-6}M, 10^{-9}M) of MBP.

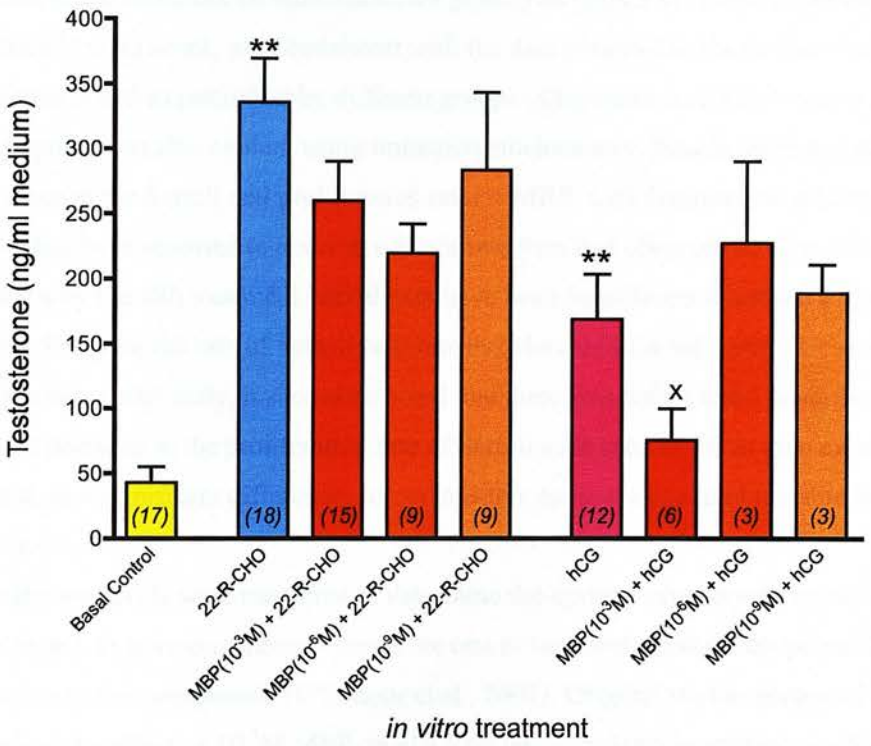

Figure 4.22: Testosterone production in vitro by e19.5 rat testis explants after culture for 48h at 37°C with or without hCG and 22-R-CHO, with or without one of three concentrations of MBP. Values are means ± S.E.M. (n values are shown in parentheses). Data were log transformed prior to analysis. Both stimulants increased testosterone production compared to untreated explants (**P<0.01) but only hCG stimulated testosterone production was significantly decreased by addition of 10^{-3}M MBP (x P<0.01).

4.4 Discussion

Previous studies (Chapter 3) demonstrated that in utero exposure to DBP caused abnormal development of the rat fetal testis including decreased production of testosterone. The primary aim of these experiments was to optimise an organotypic culture system for the rat fetal testis. Secondly, this approach examined the influence of MBP, at a maximum dose of 10^{-3}M, on testis cell morphology as well as hormone production. These results extend previous findings obtained with the in vivo exposure experimental system (Chapter 3). Analysis of testis architecture following 48h incubation of fetal rat testes demonstrated that expression of specific proteins (SMA, 3β-HSD, AMH) was still evident in the cultured tissue

with this in vitro explant culture system, as per the in vivo studies. Explants from further experiments following 10^{-3}M MBP exposure in vitro, were examined by light microscopy following immunohistochemistry. Data analysis revealed a treatment related trend towards an increase in the size of Leydig cell clusters and a treatment related trend towards an increase in the incidence of multinucleated gonocytes (MNG's). These endpoints, though not statistically significant, were consistent with the data obtained in the in vivo studies reported in Chapter 3 and as published by different groups (Kleymenova, 2005; Mahood et al., 2004). BrdU uptake was also evident using immunohistochemistry, though there was no significant difference in the Sertoli cell proliferation rates ± MBP. Cell division and differentiation in culture has been reported to occur at a lower rate than that observed in vivo, which may explain why the 48h treatment period may have been insufficient to induce a treatment related change in the rate of Sertoli cell mitosis (Merchant-Larios, 1998; Richards, 1999). Perhaps more relevantly, it should be noted that there was only a trend towards a DBP-induced decrease in the proliferation rate of Sertoli cells seen in the in vivo experiments rather than a significant difference, so perhaps this endpoint was unobtainable in the in vitro studies.

Testosterone levels were measured to determine the optimal organotypic culture regime of those tested, as hormone measurements are one of the most sensitive endpoints for studying endocrine active compounds (O'Connor et al., 2002). Original studies presented here, compared the effect of 10^{-3}M MBP on e19.5 rat testis explants in multiple studies, against untreated explants, after 48h incubation. The lack of a significant effect of 10^{-3}M MBP on basal testosterone production led to further studies. These additional experiments demonstrated that 10^{-3}M MBP could blunt the stimulatory effect of hCG on fetal testis explant testosterone production when co-administered. hCG works by binding to the cell surface receptor for luteinising hormone (LH-R), activating adenylyl cyclase and stimulating the concentrations of the second messenger cAMP which in turn mediates a multitude of intracellular activities, including an increase in the rate of steroidogenesis (Moyle et al., 1980). LH is not normally required by the testis to initiate or maintain testicular steroidogenesis until late gestation, around e20.5 in the rat, but fetal-type Leydig cells are responsive to stimulation by LH prior to detection of this hormone in plasma at e17.5 (Gangnerau and Picon, 1987).

Studies have shown that the rat placenta does not produce chorionic gonadotropin (CG) removing any source for the fetal testis (Habert and Picon, 1990). However, chronic hCG exposure used in the present in vitro studies induced a significant increase in the level of testosterone produced, demonstrating that fetal testis explants were responsive to trophic

hormone stimulation under these optimised culture conditions. It has been reported that long-term high-level exposure to hCG can cause a de-sensitisation of the adult Leydig cells to further hCG stimulation (Akingbemi et al., 2000) but other studies have shown that fetal Leydig cells are not desensitised by LH/hCG exposure (Tsai-Morris et al., 1987). Whether the explants in the present studies became insensitive to chronic hCG stimulation cannot be assessed from these experiments.

Parallel studies combining the steroidogenic intermediate 22-R-CHO with 10^{-3}M MBP produced no significant blunting of 22-R-CHO stimulated testosterone production. By providing the explants with 22-R-CHO, it was possible to bypass the start of the steroidogenic cascade to beyond the rate-limiting step at P450scc. This helped determine where in the cascade the MBP was affecting hCG stimulated steroidogenesis. Together, these studies suggest that the steroidogenic target of MBP toxicity is prior to and/ or during the mitochondrial phase of the testosterone biosynthesis cascade. Recent publications confirm that DBP and other phthalates target many of these steps (Akingbemi et al., 2004; Barlow and Foster, 2003; Parks et al., 2000; Thompson et al., 2004). These recently reported studies confirm that the absence of an effect of MBP on 22-R-CHO stimulated testosterone production was a logical result.

In the present experiments, no consistent inhibition of basal in vitro testosterone production by Ketoconazole was observed. Ketoconazole works by inhibiting steroidogenesis via suppressing activity of the various cytochrome P450 enzymes including $P450_{scc}$, 17α-hydroxylase ($P450_{c17}$) and 17,20-lyase ($P450_{c17\text{-}20}$) (Miossec et al., 1997). The dose of Ketoconazole used (100μM), was selected based on previous work carried out in our laboratory using adult human and rat Leydig cells (Qureshi 1992, PhD Thesis). Pilot studies to confirm that the concentrations of Ketoconazole used were effective should have been carried out to confirm that it was possible to induce a decrease in testosterone production in this model system, as an anticipated outcome of phthalate exposure. It could be that the fetal rat Leydig cells, as used in the present experiments, are less sensitive to Ketoconazole than adult rat Leydig cells used in previous studies (Gray et al., 1999; Powlin et al., 1998). However, as repeated studies would demonstrate there was no effect of MBP on basal testosterone production, this negative control was less critical. The effect of 100μM Ketoconazole was also investigated in fetal human testis explants as described in the next Chapter, and shown to have inhibitory effect on testosterone production.

In addition to testosterone production, the secretion of inhibin-B was measured to assess Sertoli cell function in this model system. FSH is critical for the initiation of spermatogenesis in young animals. It binds to the FSH receptor (FSH-R) which, like LH-R

activation described above, activates adenylyl cyclase and produces an increase in cAMP secretion, initiating a multitude of intracellular responses (Lloyd and Foster, 1988).
FSH treatment did not induce any increase in inhibin-B secretion, though a slight increase in testosterone production in response to FSH was observed in the overall analysis. This suggested that the Sertoli cells could be reacting to the FSH stimulation by increasing output of a signal to Leydig cells promoting an increase in testosterone production. The inverse relationship between FSH and inhibin-B seen in the adult rat was not reported in studies with neonatal rats nor could it be assessed under these experimental conditions (Sharpe et al., 1999). As the repeated studies presented here demonstrated no effect of MBP on basal inhibin-B production and as there are no recent reports of MBP having an adverse effect on inhibin-B levels in vivo, this treatment was not pursued further.

In conclusion, these data show that fetal testes can be successfully cultured under the outlined conditions for up to 48h. Rat testes dissected at this age show characteristic progression of normal development as demonstrated by continuing testosterone production and Sertoli cell proliferation. However, not all of the adverse effects on rat fetal testis development induced by in utero DBP exposure were successfully modeled using primary cultures of fetal rat testis explants, exposed in vitro to the active DBP metabolite MBP. This suggests that this approach has only limited utility for investigating the changes in fetal testis development associated with DBP exposure.
Additional studies, using the same culture regime, were carried out but with fetal human testis explants and the results are described in Chapter 5.
In order to confirm whether 48h exposure to MBP in vitro was sufficient to induce the range of changes seen following 8 days of DBP-treatment in vivo, new in vivo studies involving just 48h of DBP exposure from e19.5-e21.5 were performed and data compared with those obtained using treatment from e13.5-e21.5. These data are reported in Chapter 6.

5 Studies using fetal human testis explants

Studies using the human fetal testis, parallel to those performed in the rat fetal testis (Chapter 4), would theoretically enable a comparison of the relative sensitivity of the two species to the DBP metabolite MBP, contributing to the assessment of the risk that phthalates pose to human reproductive health.

5.1 Introduction

At the time when these studies were initiated, the range of published work that described the effects of DBP on fetal testis development largely examined the adverse effects of phthalate administration on fetal rats via maternal exposure. There was little work describing fetal human exposure to phthalates directly but there were reports of adult human urine containing higher than anticipated levels of phthalate metabolites (Blount et al., 2000a).They measured the phthalate metabolite levels in the urine rather than parent compound levels in serum for these main reasons:

- previous studies that measured total phthalate di-ester in serum were fraught with technical problems, such as contamination as a consequence of ubiquitous exposure to the parent compound, which masked low level measurements in samples (Harvan et al., 1980; Luster et al., 1978).
- phthalate mono-esters only exist as a consequence of metabolism and do not bioaccumulate so act as accurate biomarkers of recent di-ester exposure. Metabolism of the di-esters is rapid in humans and growing evidence hints that the primary metabolites, the mono-esters, rather than the parent compounds, induce the toxic effects that are seen (Foster et al., 1983; Saillenfait et al., 1998).

One outcome of the Blount studies was the observation that though DBP was not the phthalate produced in the greatest quantities (which is DEHP), it was being internalised by humans at a much higher levels than anticipated (Blount et al., 2000b). This has since been supported by additional studies where the estimated DBP intake by Germans = 16.2μg/kg bodyweight per day compared to DEHP at 13.8μg/kg bodyweight per day (Koch et al., 2003). These data suggested that internalisation of DBP was much greater than previously thought. However, it should be noted that the low urine measurements of the metabolites of DEHP (e.g. MEHP) could be the consequence of low exposure but also of storage in adipose tissue or excretion via an alternative route e.g. faecal secretion for these more lipophilic compounds. The MEHP levels measured were consistent with those reported previously in the urine after occupational exposure (Dirven et al., 1993).

Interestingly, a sub-population of people, predominantly women of child-bearing age, had urinary MBP levels in the range 312-2763μg MBP/g creatinine, whereas the majority of the samples had <60μg MBP/g creatinine (Blount et al., 2000b). This is of particular concern to

public health given the reproductive potential of the sub-population at greatest exposure and the published reports of reproductive toxicity seen in DBP exposed laboratory animals. More research into the developmental effects of phthalates on fetal development, especially by DBP was called for.

5.1.1 The in vitro approach

In order to make useful comparisons between the in vitro findings with the rat and human fetal testis explants, a comparison of the development of the testis in the two species was appropriate in order to highlight their similarities and differences (see Chapter 1 for more detail).

For both species, gonadal differentiation may be divided into four stages: pregonadal, indifferent, primary sex differentiation, and secondary sex differentiation (Voutilainen, 1992). By week six of gestation (6/40w) in the human (e12 in the rat), the hitherto indifferent XY gonad starts to differentiate and move from its position high in the abdominal cavity to become a complex organ, the testis positioned outside the abdominal cavity in the scrotum (Malas et al., 1999). The testis of both species has two major roles: the production of male gametes, accompanied by hormone production, primarily testosterone. The biggest difference in testis development between the two species is the timing. In the rat, the initiation of gonadal development is postponed until the mid-trimester, of the 22-day embryonic period (e). In the human, there is a 40-week (/40w) gestation period and gonadal differentiation is initiated around 6/40w then progresses steadily throughout the pregnancy. However, the extended time of development in the human may mean that there is prolonged vulnerability to any adverse effects of phthalate exposure. It may also mean that the maximal duration of in vitro exposure of testis explants is insufficient to provoke the same level of response in the human tissue as seen in the rat tissue, due to the differences in development rate, i.e. slower in the human.

5.1.2 Experimental Objectives

The aim of these studies was to set up a culture system allowing normal fetal testis development to occur in vitro and then investigate whether normal development was disrupted after in vitro exposure to phthalates. For this purpose, the development/ function of the testis explants was assessed by three main criteria, as was done for the optimisation of the fetal rat testis in vitro system (Chapter 4):

- maintenance of tissue architecture and protein expression
- analysis of the somatic cell proliferation rate
- measurement of hormone production ± stimulation/ inhibition

These three criteria would also be used to compare the endpoints seen in the human fetal testis explants with those seen in the rat fetal testis experiments (Chapter 4).

In vitro incubation of human testis explants at different fetal ages will provide quantitative information regarding their steroidogenic potential, their sensitivity to known steroidogenic agents and susceptibility to the effects of MBP treatment. Moreover, these experiments exploit the only means by which human tissue can be exposed to phthalates at known concentrations for designated durations. This is a unique method by which meaningful information about the effects of direct MBP exposure on fetal testis development can be obtained.

5.2 Materials and Methods

Further details on the general materials and methods used are listed in Chapter 2. Overall, following completion of the incubation, media were stored at -20°C before being assayed for steroid production and explants were analysed for histological changes. The methods used were consistent with the procedures developed for the experiments with fetal rat testis explants in Chapter 4 with any species specific differences outlined below.

5.2.1 Sampling of human fetal testes

In November 2004, the Human Tissue Act was introduced, in most part due to the public enquiries held at Alder Hey and Bristol Royal Infirmary hospitals. This Act provided new legislation covering the removal, storage and use of human organs and tissue, from dead and living people. It does not come into affect until 2006 and even then will not apply in Scotland, where this research was performed. However, the Medical Research Council (MRC), that funded my work, had previously issued a set of guidelines: "Human Tissue and Biological Samples for use in Research" (April 2001), primarily aimed at its relevant researchers and ethics committees. It is under the MRC 2001 guidelines that these studies were conducted. It should be noted that even with the Act in place, embryos outside the human body are excluded from its governance.

5.2.1.1 Explant preparation

Fetal human testes were collected and explants prepared as detailed in Chapter 2. At least one pair of testes per gestational week was stored (one after fixation in Bouin's, the other intact at –80°C) and not dissected into explants for culture.

Testis explants were cultured on a 0.45μm pore size culture insert with tripod scaffold (Millipore, UK), secured in a well of a 24-well tissue culture plate, as previously described and as in Chapters 2 and 4 (Habert et al., 1991). Up to six explants were placed carefully on the filter and covered in 0.2ml medium. A further 0.2ml of medium was placed between the

well and the scaffold. Explants were incubated at 37°C in an atmosphere of 5% CO_2 for up to 48h. At least one explant was always fixed in Bouin's prior to incubation, as a baseline control (t=0h).

5.2.2 Explant culture conditions

The same in vitro conditions were used to incubate the fetal human testis explants as were optimised for the fetal rat testis explant experiments detailed in section 4.1.1.1.

5.2.2.1 In vitro treatments

In order for the incubated testis explants to be exposed to specific treatments, the chemicals were mixed with the culture medium prior to addition to the culture well and incubation of the testis explants. The treatments used, their preparation and dose levels, are detailed in Chapter 2. Medium was not changed during the experiment but was collected at the end and stored at –20°C until assayed. At the end of the experiment, explants were fixed in Bouin's for 1h and then processed into paraffin blocks or snap frozen and stored at –80°C.

5.2.3 Analysis of testis architecture and protein expression

Up to six explants were incubated on a single filter, in a single well. Explants from the same well were fixed and embedded together in the same paraffin block. Each block was serially sectioned (5μm slices), mounted on numbered slides then stained and subjected to analysis as a single replicate. Explants that were fixed in Bouin's were examined microscopically after immunohistological processing. Biological markers were used to reveal the explant morphology. Markers used in these experiments are detailed below.

5.2.3.1 Distribution of peritubular myoid cells

Formation of the seminiferous cord requires peritubular myoid cells (Cupp et al., 2003). These could not be visualised using immunostaining for the cytoskeletal protein Smooth Muscle Actin-α (SMA) as used for the experiments in Chapter 4, as the human peritubular myoid cells did not express this protein, though they could be recognised morphologically, and the expression of this SMA protein was observed in blood vessels (Figure 5.0). It has been reported that SMA-α is marker of terminal differentiation of smooth muscle cells in the rat testis so it maybe that these cells were undergoing differentiation in the fetal human testis (Palombi et al., 1992). Therefore, possible treatment effects of SMA distribution between species could not be compared.

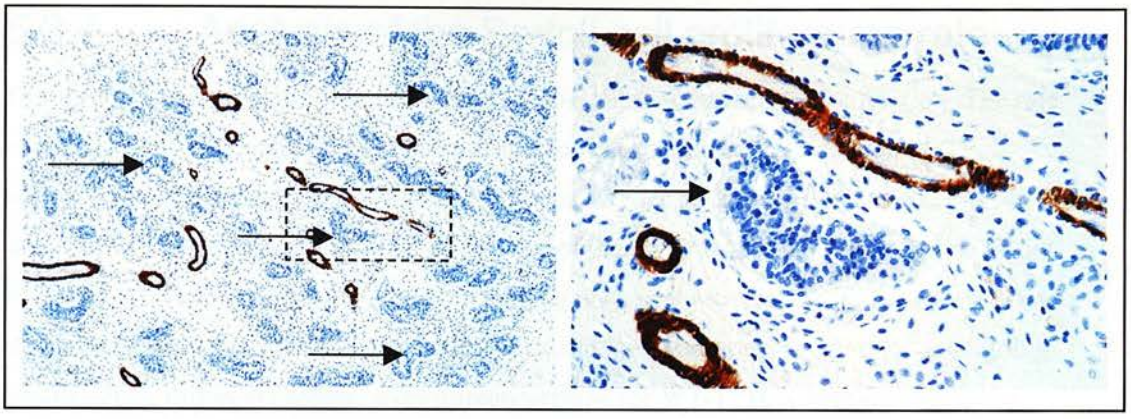

Figure 5.0: SMA immunostained sections of human fetal testis explants without in vitro culture. Note that the blood vessel walls are stained (brow) but cord perimeters (locale of the peritubular myoid cells) are immunonegative (arrows). Note the scattered distribution of the interstitial cells (including Leydig cells) The left panel was photographed using a x10 objective and a focal area re-photographed (rectangle) with a x40 objective as shown in the right panel.

5.2.3.2 Quantification of Leydig cell distribution

Three numbered slides per block (at least five serial sections apart) were deparaffinised, rehydrated and immunostained for 3β-HSD (3β-HSD antibody gifted by Professor JI Mason, University of Edinburgh) and lightly counterstained with haematoxylin, according to the protocol described in Chapter 2. The total area of stained tissue per slide was measured and the distribution and size of brown clusters (immunopositive Leydig cells) within the outlined blue (counterstained) area, was quantified using a light microscope with a x63 objective and computer-assisted image analysis as described in detail in Chapter 2 and similar to that published for the rat (Mahood et al., 2005).

5.2.3.3 Quantification of multinucleated gonocytes

One slide per block was deparaffinised, rehydrated and immunostained for AMH using a goat anti-AMH polyclonal antibody (Santa Cruz) and counterstained with haematoxylin, according to the protocol described in Chapter 2. The large spherical nuclei of the gonocytes were easy to distinguish, amongst the AMH stained Sertoli cell cytoplasm, using a light microscope with a x63 objective, as were the counterstained perimeters of the nuclear membrane(s). The number of seminiferous cords per cross section was counted and each cord assessed for containing none or at least one gonocyte with >1 nuclei per cytoplasm membrane (a multinucleated gonocyte). The percentage of cords containing at least one multinucleated gonocyte (MNG) was calculated per block.

5.2.4 Analysis of the Sertoli cell proliferation rate

During fetal testis development, the various testis cell types undergo proliferation. The rate of proliferation can be measured by exposing the testes to the nucleotide base dUTP, supplied in vitro as the parent compound 5-Bromo-2'-deoxyuridine (BrdU), which is incorporated into the reproduced DNA in place of the dTTP base. The incorporation of the dUTP base can be visualised by immunostaining with a relevant antibody. In order to ensure that proliferation was quantified in Sertoli cells and not another testis cell type undergoing proliferation, different sections were immunostained for WT-1. This staining within the tubules was exclusively localised to the Sertoli cells and any extratubular staining was undefined.

The incidence of Sertoli cell apoptosis was also investigated by labelling fragmenting (apoptotic) DNA by catalytic incorporation of fluorescein-12-dUTP at the 3'-OH ends of the DNA using the terminal deoxynucleotidyl transferase enzyme. This method could discern apoptotic cells from cells undergoing necrosis, also recognisable microscopically by their darkly counterstained pyknotic nuclei.

5.2.4.1 Quantification of BrdU incorporation index

Measurement of the 5-Bromo-2'-deoxyuridine (BrdU) incorporation index was possible after the cultured testis explants were treated with BrdU during the last 4h of the culture as based on Livera et al (2000). Three numbered slides per block (at least five serial sections apart) were deparaffinised, rehydrated and immunostained for BrdU incorporation using a sheep anti-BrdU polyclonal antibody (Fitzgerald Laboratories) and counterstained with haematoxylin, according to the protocol described in Chapter 2. The number of Sertoli cell nuclei that showed a clear positive immunoreaction to BrdU per 1000 counted, using a light microscope with a x63 objective, was converted to a percentage i.e. the BrdU incorporation index. As BrdU was only incorporated in cells during proliferation, this value was also referred to as the proliferation index (PI). A mean from each block was calculated and at least seven blocks per treatment were analysed.

5.2.4.2 Quantification of apoptosis

Apoptotic cells were detected in situ by use of the TUNEL method as described by Livera et al (2000). There were no TUNEL positive (apoptotic) stained cells seen in the human fetal testis sections investigated. The positive control sections (rat testis, d18) did work which confirmed that the protocol was followed successfully (see Chapter 2).

5.2.5 Analysis of hormone secretions

Hormone measurements are one of the most sensitive endpoints for studying endocrine active compounds (O'Connor et al., 2002). To ensure that the explant culture system was viable, four separate treatments were added to the media to manipulate the testosterone production level of the testis explants. Testosterone production was manipulated by specific treatments and their effects were compared to basal hormone production levels as measured in the media of untreated explants. More details on the doses and rationale behind these choices are provided in Chapter 2.

5.2.5.1 Measurement of testosterone production

The testosterone secreted into the media was measured in duplicate by radioimmunoassay (RIA) as described in Chapter 2. No extraction was performed as the only steroid that, in addition to testosterone, significantly cross-reacts with the testosterone antibody (17β-hydroxy-5α-androstane-3-one) is secreted in minute amounts by the fetal testis (Habert and Picon, 1984). Typically, a 1/10,000 dilution of collected culture media was prepared for assay.

5.2.5.2 Measurement of inhibin-B production

Though the human fetal testis is able to synthesize and secrete inhibins, previous experiments (Chapters 3 and 4) showed no significant effects of any treatment on inhibin-B production levels, so the focus of these studies centred on effects of treatment on testosterone production and inhibin-B data was not generated (Voutilainen, 1992).

5.2.6 Phthalate Exposure

In the previous Chapter, explants of fetal rat testes were incubated with the DBP metabolite MBP at doses of up to 10^{-3}M. Parallel studies were conducted with fetal human testis explants and the results are presented in this Chapter.

The details of the mechanism by which phthalates induce change in the development of the rodent fetal testis is unclear, but appears to be associated with Leydig cell testosterone biosynthesis (Akingbemi et al., 2001). As with the rat studies, fetal human testis explants were exposed to MBP together with the steroidogenic stimulants hCG or 22-R-CHO which stimulate testosterone production by acting at different points in the steroidogenic cascade. By comparing whether the steroidogenesis stimulants exerted a reduced effect on testosterone production with MBP present, it could be pinpointed where in the steroidogenic pathway the DBP metabolite was having its inhibitory effect. These experiments were repeated with different concentrations of MBP to investigate any dose response effect.

5.2.7 Statistical Analysis

For analysis of the testosterone production data, the mean level per treatment was determined for each experiment. These values were plotted and subject to statistical analysis (ANOVA, Dunnett's test). Additionally, the relative change in mean testosterone production per treatment was plotted, expressed as a percentage of the basal production per experiment and subject to statistical analysis (ANOVA, Dunnett's test). Where the format of the data affected the statistical significance of the treatments, the two plots are presented together. For all data, where sample size was less than 3 (i.e. 1 or 2), the range of the data (standard deviation from the mean (S.D.)) was plotted, rather than the standard error from the mean (S.E.M.), unless otherwise stated. A sample size of less than three was not subjected to statistical analysis. It should be noted that any differences between treatment groups might be the consequence of the small sample pool. A small sample size (n) is more vulnerable to being skewed by any extreme responses by outlier data points. Alternatively, because samples at different developmental ages were pooled, age specific effects of treatment may have been diluted by the varying sensitivity of differently aged samples. The pooling approach was considered appropriate as the aim of the studies was to try to induce gross changes in testis architecture and hormone production with treatments, to compare with the results from parallel experiments with the rat fetal testis, described in Chapter 4.

5.3 Results

The in vitro methods used for the culture of the available human fetal testes were based on the optimised rat model as detailed in Chapter 4. Availability of human fetal samples was sporadic and samples were of unpredictable fetal age. For presentation and purposes of statistical analysis, data of all ages was pooled per treatment prior to analysis, unless stated otherwise.

5.3.1 Age effect on testis weight and testosterone production

The effect of human fetal age at the time of testis explant preparation was investigated. Changes in testis weight and testosterone production with increasing age were compared between 2nd trimester fetal human testes, aged between 14-20w/40w.

Testes were weighed following retrieval from the body cavity and removal of remaining non-testicular tissue. Testis weight increased with fetal age and linear trend analysis showed that this was highly significant ($P<0.0001$) (Figure 5.1). The increase in testis weight correlated with the number of explants that could be generated per testis (data not shown), as seen with the rat tissue (Table 4.1).

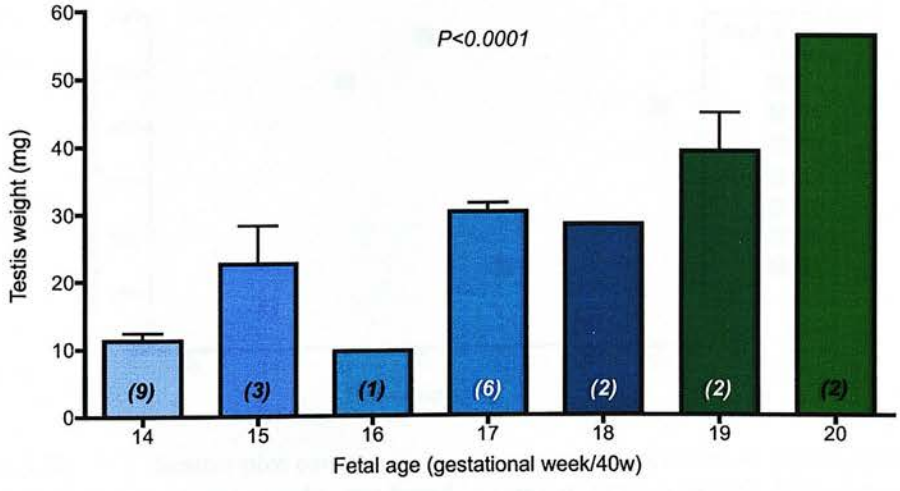

Figure 5.1: Testis weight by human fetal age (gestational week/40w). Values are means where n=2 and means ± S.E.M. where n>2 (n values are shown in parentheses). Testis weight increased significantly with gestational age.

The level of basal testosterone produced over 48h during in vitro incubation by testis explants retrieved from fetuses aged 14-20w was compared. Mean testosterone production levels rose with fetal age from 14w to a peak at 17w (74 and 558 ng testosterone/ml medium, respectively), then dropped over 18-20w achieving a mean production level of 92 ng

testosterone/ ml media (Figure 5.2a). The rise and fall in testosterone production with fetal age seen in vitro, mimicked the previously reported pattern of fetal human testis testosterone production in vivo (Voutilainen, 1992). There was no correlation seen between testis weight (mg) and the basal level of testosterone produced (Figure 5.2b).

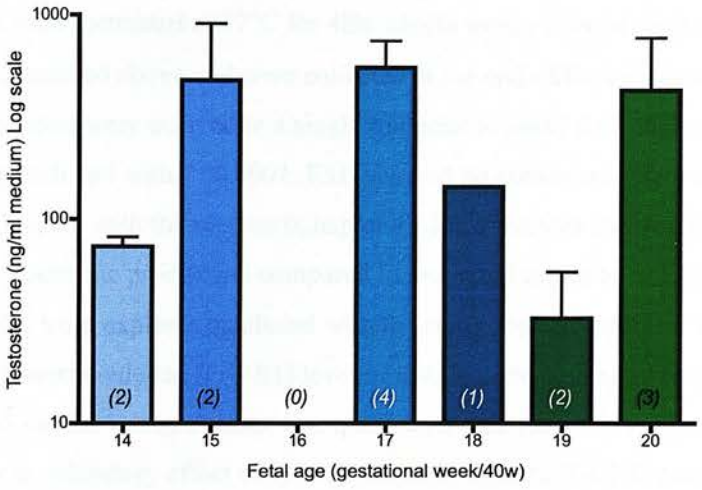

Figure 5.2a: Basal testosterone production by explants of human testis from fetuses of increasing ages, incubated in vitro at 37°C, over 48h. Values are means where n=1-2 and means ± S.E.M. where n>2 (n values are shown in parentheses). There was no significant effect of fetal age on in vitro testosterone production but production peaked at 17w.

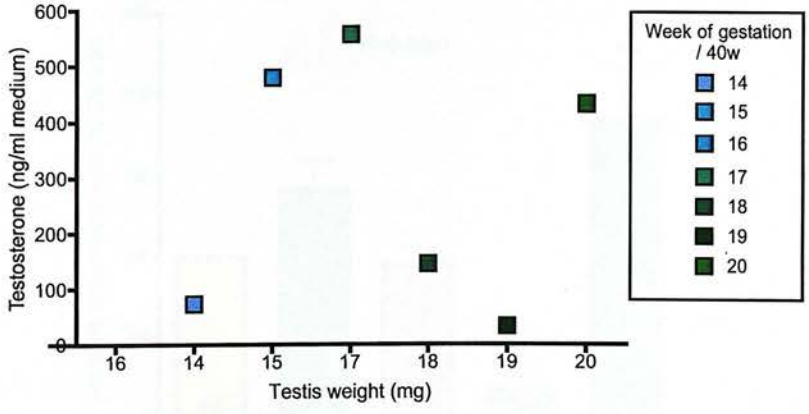

Figure 5.2b: Scatter plot comparing basal testosterone production levels by explants of human testis of increasing weight, incubated in vitro at 37°C, over 48h. Values are means of the data in Figure 5.2a. There was no significant trend between testis weight and the level of basal testosterone production.

Note: due to the small number of testes that were available and their varying ages, assay results were adjusted per testis (per experiment) as a relative amount (%) of the basal testosterone production level. This meant that the basal value would always be 100%.

5.3.2 Manipulation of testosterone secretion in vitro

Testis explants were exposed to four agents, three of which are known to affect steroidogenesis, to investigate whether testosterone production could be manipulated. Further details on these compounds are provided in Chapter 2.

Testis explants were incubated at 37°C for 48h. Media were prepared with or without the four additions described above and were collected at the end of the experiment. Samples from multiple studies were assayed in a single run prior to assay data analysis (Figure 5.3). ANOVA was significant with P<0.0001. FSH showed no consistent effect on testosterone production, consistent with the rat data (Chapter 4). Data analysis showed no effect of hCG treatment on testosterone production compared to untreated explants, unlike seen with the rat tissue. The media from explants incubated with the steroidogenic inhibitor KTZ consistently showed a significantly reduced (P<0.01) level of testosterone compared to untreated explants. This decrease was in contrast to experiments with rat testis explants, which were unable to show an inhibitory effect of KTZ. Treatment with 22-R-CHO consistently increased testosterone production relative to basal levels consistent with the rat data (Chapter 4). Overall, these results suggest that steroidogenesis in the fetal testis of the two species is sensitive to different levels of stimulation and inhibition.

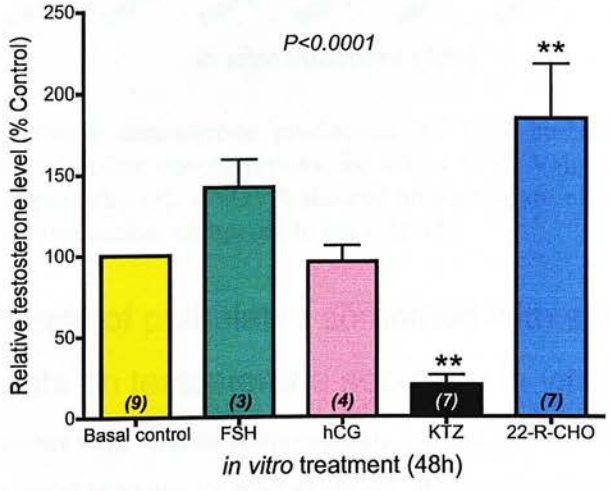

Figure 5.3: Production of testosterone in vitro by fetal human testis explants aged between 14w-20w gestation, after culture for 48h with different media additions. Values are means ± S.E.M. (n values are shown in parentheses). KTZ significantly reduced testosterone production (**P<0.01) and 22-R-CHO significantly increased testosterone production (**P<0.01) when data were analysed as relative values. There was no significant effect of hCG or FSH.

5.3.3 Effect of DBP and MBP on testis explants

5.3.3.1 Effect of DBP or MBP on testosterone production

Analysis of testosterone levels, measured in media from phthalate treated explants, showed no effect of increasing phthalate concentration on testosterone production, compared to basal levels ($P>0.78$, Figure 5.4). This finding was consistent with the fetal rat testis explant experiments (section 4.1.3.1).

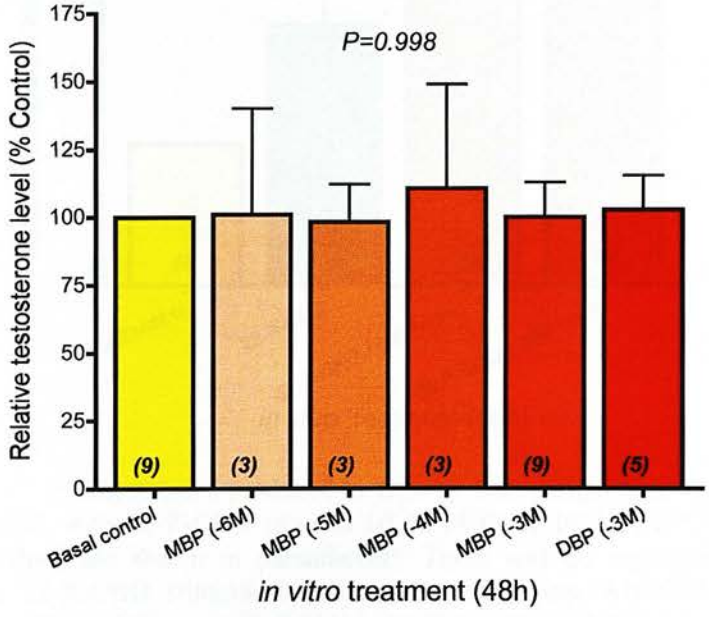

Figure 5.4: Relative testosterone production by fetal human testis explants after culture with different phthalate concentrations, for 48h at 37°C. Values are mean ± S.E.M. (n values are shown in parentheses). ANOVA showed no significant effect of neither MBP nor DBP on testosterone production, compared to basal levels.

5.3.3.2 Effects of phthalates combined with steroidogenic stimulants on testosterone secretion in vitro

In previous experiments with fetal rat testis explants, neither MBP nor DBP had an inhibitory effect on basal testosterone production but MBP co-incubated with hCG did blunt the stimulatory effect of hCG alone on testosterone production. No effect of MBP on 22-R-CHO stimulated testosterone production was seen in the fetal rat experiments. These experiments were repeated using fetal human testis explants, incubated with a combination of 22-R-CHO or hCG plus 10^{-5}M MBP or 10^{-3}M MBP or 10^{-3}M DBP. Testosterone data were compared against basal and stimulant treated levels.

The level of 22-R-CHO stimulated testosterone output was not significantly reduced when either MBP or DBP were co-administered (Figure 5.5). This was consistent with previous

experiments (Chapter 4) in which no significant effect of MBP on testosterone production by fetal rat testis explants was seen when administered alone or co-administered with 22-R-CHO.

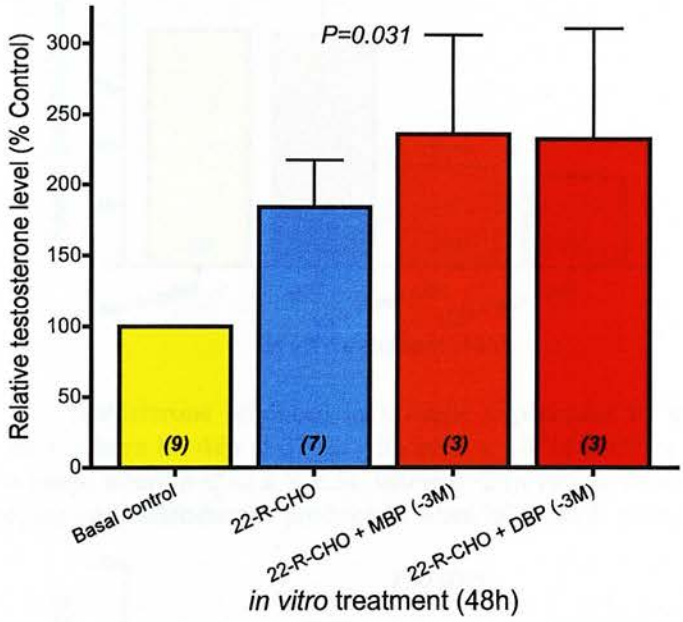

Figure 5.5: Testosterone produced in vitro by fetal human testis explants after culture for 48h at 37°C, with 22-R-CHO (R) and 10^{-3}M MBP or 10^{-3}M DBP. Values are mean ± S.E.M. (n values are shown in parentheses). There was no significant effect of either phthalate on 22-R-CHO stimulated testosterone production. ANOVA of relative levels showed a significant difference (P=0.031) in testosterone production between basal and all 22-R-CHO incubated explants.

Different explants were incubated with a combination of hCG plus 10^{-5}M MBP or 10^{-3}M MBP. Testosterone production data from co-incubated explants were compared against levels of testosterone production from explants incubated with just hCG. Co-incubation of the explants with hCG in a single experiment revealed a trend towards a reduction in the amount of testosterone produced with both concentrations of MBP (Figure 5.6a). When the individual co-incubation data were analysed against the pooled basal and hCG data (as in Figure 5.3), then a significant difference was achieved (P<0.01) (Figure 5.6b).

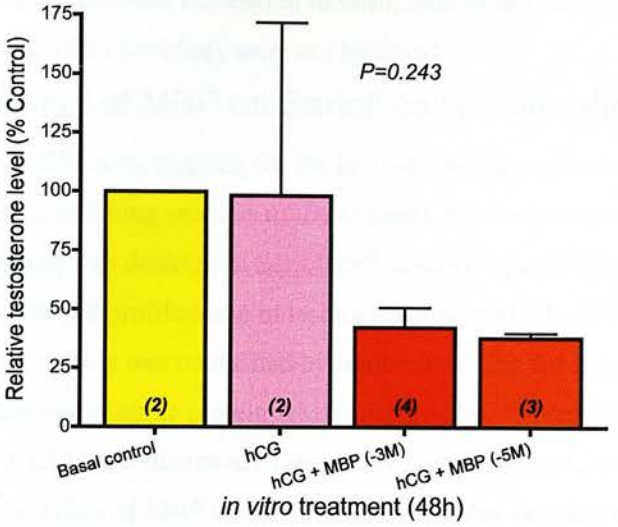

Figure 5.6a: Testosterone produced in a single experiment by explants from a fetal human testis after culture for 48h at 37°C, with hCG ± 10^{-5}M MBP or 10^{-3}M MBP. Values show a mean ± range where n=2 or ± S.E.M. where n>2 (n values are shown in parentheses). There was a reduction in testosterone production when hCG/MBP were co-incubated.

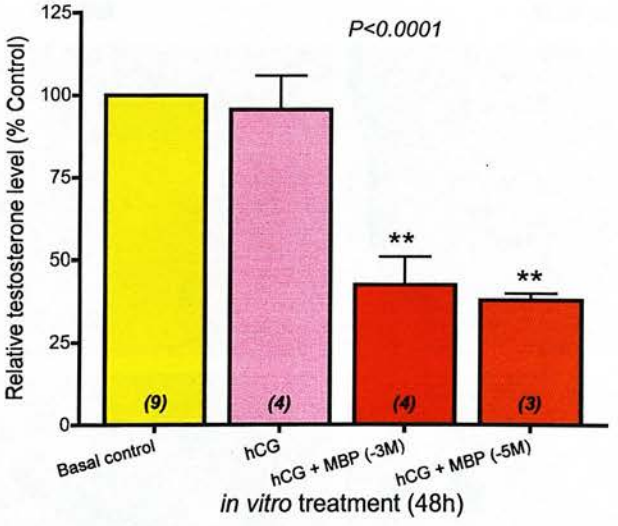

Figure 5.6b: Testosterone produced by fetal human testis explants after culture for 48h at 37°C, with hCG ± 10^{-5}M or 10^{-3}M MBP. Relative basal and hCG values from multiple experiments were plotted with the co-incubation data from Figure 5.6a. ANOVA = P<0.0001 and further analysis showed that co-incubation with 10^{-5}M or 10^{-3}M MBP significantly (**P<0.01) reduced testosterone production to below basal levels.

5.3.3.3 Effects of MBP on testis cell histology

It was hypothesised that in vitro incubation of human fetal testis explants, exposed to MBP, might induce similar histological changes to those seen with rat fetal testis explants incubated with MBP (Chapter 4). For comparison, the same parameters were assessed in

fetal human testis explants after incubation in vitro, with or without 10^{-3}M MBP. The effects of DBP in vitro on explant histology were not assessed.

5.3.3.3.1 Effect of MBP on Sertoli cell proliferation

The level of cell proliferation ongoing for the last 6-8h of incubation of the experiment was assessed after immunostaining sections of the explants, for the incorporation of BrdU. No BrdU immunostaining was detected in Sertoli cell nuclei (Figure 5.7) so it was concluded that Sertoli cells were not proliferating in testis explants, aged 14-19/40w. The expression of known Sertoli cell proteins was confirmed by immunostaining for the nuclear protein WT-1 (Figure 5.8) and the cytoplasmic protein AMH (Figure 5.9). As Sertoli cells were the only cell type for which a PI was calculated in the rat testis experiments, both in vivo and in vitro, a comparison of the effect of MBP on the proliferation index between the species could not be made. The proliferation indices of non-Sertoli cells were not determined but this observation was consistent with previous studies of ex-vivo human testes across a similar age range (Murray et al., 2000; Voutilainen, 1992).

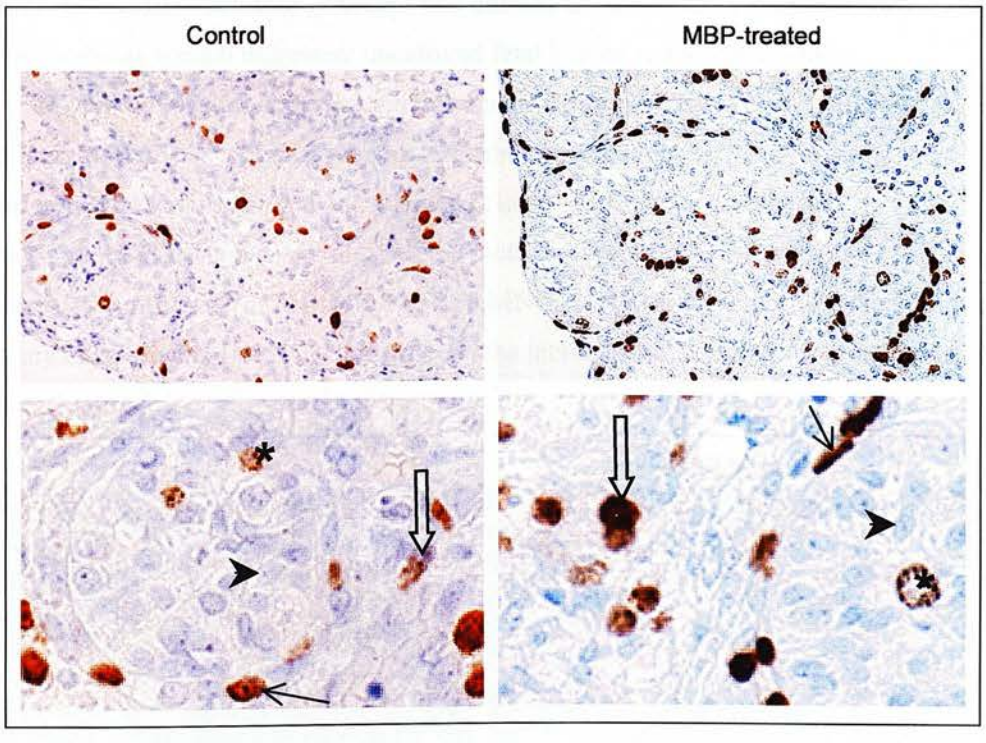

Figure 5.7: BrdU immunostained section of fetal human testis explants after culture for 48h, with or without MBP (10^{-3}M). BrdU was incorporated by peritubular myoid cells (narrow arrows), interstitial cells (wide arrows) and round intratubular germ cells (asterix) but no intratubular Sertoli cells (arrow heads). The top panels were photographed using a x40 objective with focal areas enlarged in the panels below. Any difference in colouration is an artefact of the image processing and was not evident visually.

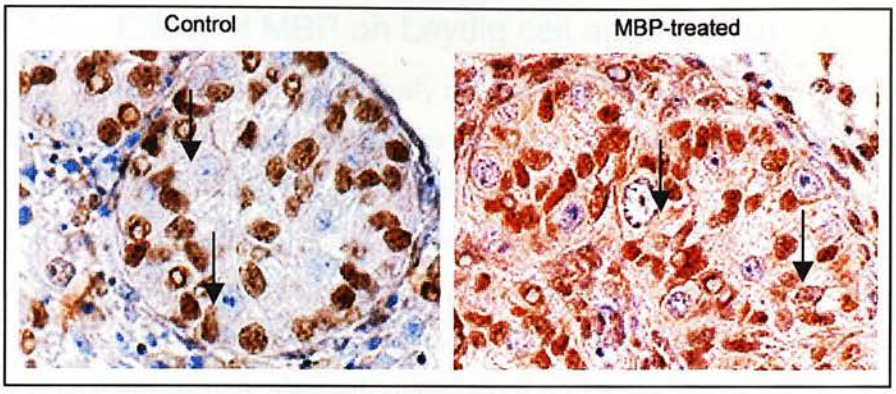

Figure 5.8: WT-1 immunostained section of fetal human testis explants after culture for 48h, ± MBP (10^{-3}M). WT-1 is used as a marker of intratubular Sertoli cell nuclei (brown); WT-1 negative intratubular cells are most likely to be fetal germ cells (arrow). These panels are focal areas enlarged from images photographed using a x40 objective.

5.3.3.3.2 Effect of MBP on gonocytes

Gonocytes were undergoing normal mitotic proliferation during the fetal ages examined, hence an abnormally nucleated gonocyte was defined as having more than two nuclei per cell membrane as normal untreated/ uncultured fetal human testis cords contain mononucleated gonocytes, with rare and infrequent multinucleated gonocytes (MNG's). AMH immunostained sections from cultured explants were analysed and the percentage of seminiferous cords per section that contained at least one MNG was recorded.

Only a single MNG was seen amongst all the sections examined, in an untreated explant, therefore the significance of MBP exposure on MNG incidence could not be evaluated nor could any comparison of the effect of MBP on the incidence of MNG's, between the species, be made (Figure 5.10).

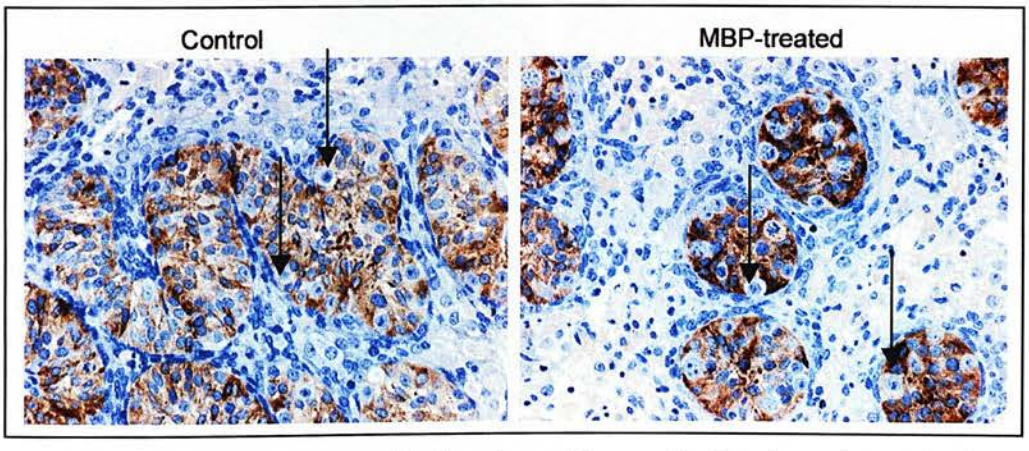

Figure 5.9: AMH immunostained sections of human fetal testis explants after in vitro culture ± MBP (10^{-3}M), for 48h at 37°C. Note that gonocyte nuclei are mononuclear (arrows) and surrounded by stained Sertoli cell cytoplasm. There was no discernible effect on the gonocyte nuclei with MBP-treatment. These panels show cross sections through testis explants photographed using a x40 objective.

5.3.3.3.3 Effect of MBP on Leydig cell aggregation

Leydig cell aggregation has been previously measured in the rat (Chapters 3 and 4) using a computer assisted stereological method, after fixed testis sections were immunostained for the steroidogenic enzyme 3β-HSD (3β-hydroxysteroid dehydrogenase), to highlight Leydig cell distribution. Compared to the fetal rat testis sections analysed in the previous two Chapters, the distribution of the Leydig cells in the fetal human testis explants was scattered (Figures 5.0 and 5.10) rather than aggregated (Figure 4.20), prohibiting computer assisted analysis of their distribution. Alternative Leydig cell markers to 3β-HSD were investigated, including P450scc, StAR and SR-B1 (not shown) but none of these could be "read" by the computer software either. This meant that the Leydig cell distribution of in vitro cultured human fetal testis explants could not be successfully quantified. Qualitative assessment showed no obvious difference in Leydig cell distribution between untreated explants or those treated with MBP and there was no obvious, reproducible, effect of treatment on the intensity of the immunostaining achieved with any of the Leydig cell markers tried. However, in support of the present experiments, the testicular steroidogenic enzyme expression data generated in these studies, correlated well with previously reported changes in gonadal steroidogenesis with human gestational age (Voutilainen and Miller, 1986).

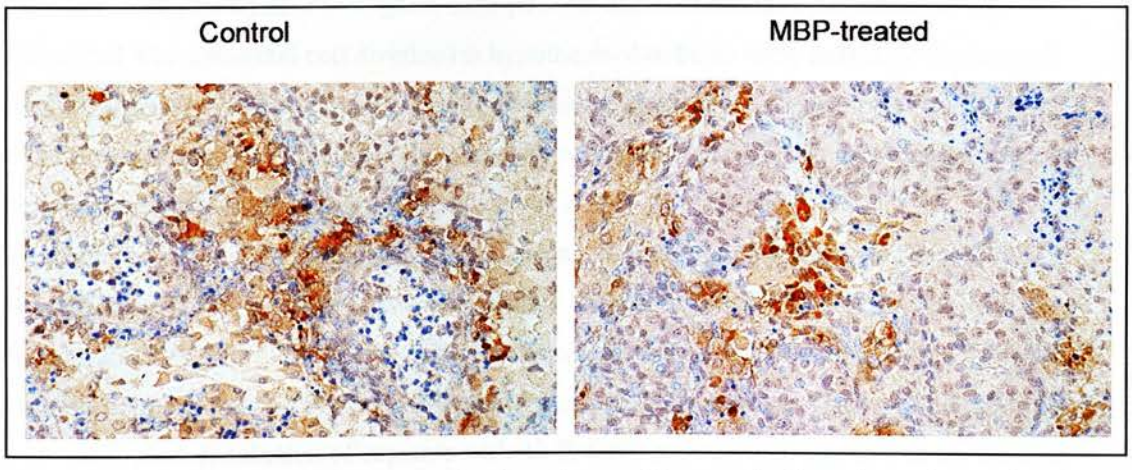

Figure 5.10: 3β-HSD immunostained sections of human fetal testis explants after in vitro culture with or without MBP(10^{-3}M), for 48h at 37^{0}C. Note the dispersed arrangement of the Leydig cells (brown). There was no discernible change in Leydig cell distribution with MBP-treatment, compared to the control explants. These panels show cross sections through testis explants photographed using a x40 objective.

5.4 Discussion

Previous studies (Chapters 3 and 4) were able to demonstrate that exposure to DBP in utero or MBP in vitro caused abnormal development of the rat fetal testis, including decreased

production of testosterone. The primary aim of these experiments was to use an in vitro organotypic culture system for the human fetal testis to investigate the influence of MBP, at a maximum dose of 10^{-3}M, on testosterone production and testis development. Whether fetal age affected their sensitivity, could not be assessed from these experiments.

Analysis of testis architecture and cell proliferation in fetal human testes, demonstrated that protein expression indicative of the cell distribution, did differ from the fetal rat testis explants, even for untreated samples. In the human explants, there was little if any staining of the peritubular myoid cells with SMA, used to highlight the perimeters of the developing seminiferous cords, and the Leydig cells were less aggregated, prohibiting cluster analysis. These were not artefacts of the culture process as they were evident in uncultured tissue (not shown). The Sertoli cells were not proliferating at all, though other testis cell types were, including the fetal germ cells, again in contrast to the findings with the fetal rat testis cultures (Murray et al., 2000). This observation was consistent with previous observations of uncultured human testes across this age range (Murray et al., 2000). The viability of the Sertoli cells was confirmed by the expression of the proteins WT-1 and AMH, as necrotic cells present in the cultured tissue did not express cell-specific proteins (Sharpe et al., 2003). The absence of multinucleated gonocytes in the treated samples was different from the rat in vitro experiments where MNG's were found in all cultured explants, with or without MBP exposure. Polyploidism, of fetal germ cells (though different to multi-nucleation is still associated with abnormal cell division) is hypothesised to be an early indicator of germ cell tumourigenesis so though it may not have been induced in these samples under these conditions, it may yet be inducible by phthalate exposure over different timepoint, dose level or exposure duration and in vivo (Chemes et al., 2003).

Testosterone production was measured as a means of determining whether MBP (10^{-6}M, 10^{-5}M, 10^{-4}M or 10^{-3}M) had any effects comparable to those found in the experiments with the fetal rat testis explants (Chapter 4). There was no effect of up to 10^{-3}M MBP or 10^{-3}M DBP on basal testosterone production by the fetal human testis explants, consistent with the in vitro rat studies. Incubation of explants with 22-R-CHO produced an increase in the level of testosterone produced, also consistent with the in vitro rat studies.

Experiments in which explants were incubated with the steroidogenic stimulant hCG had no effect upon basal testosterone production. This was contrary to the idea that placental hCG may regulate testosterone synthesis during this phase of gestation (Voutilainen, 1992). Additional studies where fetal testicular testosterone production was measured following long term, high dose gonadotropic stimulation of cultured human, rhesus monkey, and rabbit fetal testes, reported that fetal Leydig cells were capable of responding to gonadotropins (0-

100 ng/ml hCG) and could secrete testosterone at high levels for up to 24h (Leinonen and Jaffe, 1985). Further studies also demonstrated the role of hCG in the regulation of testicular steroid production in human fetuses from 14 to 20 weeks gestational age using saturable binding of ^{125}I-hCG to testicular homogenates. The greatest increase in testosterone production occurred when the hCG concentration was increased from 0.5 to 5 ng/ml (= 0.005 to 0.05iu (physiologically relevant range, compared to the 0.1iu/ml used in the present studies (Huhtaniemi et al., 1977). However, Word et al (1989) were unable to show a response to hCG stimulation in homogenates of fetal testes obtained from first and second trimester human abortuses, consistent with the present studies. These findings suggest that it is unclear whether the onset of testosterone formation in human fetal testes is independent of gonadotropin control (Word et al., 1989).

Additional experiments involved incubating explants with a mixture of hCG and MBP (10^{-5}M or 10^{-3}M) and revealed that both doses of MBP reduced the amount of testosterone produced compared to when explants were incubated with hCG alone. This result was consistent with the rat in vitro experiments. Though the human explants appeared to be sensitive to MBP at a lower concentration (10^{-5}M) than the in vitro rat experiments when co-incubated with hCG, the rat explants were exposed to MBP at slightly different concentrations of (10^{-6}M or 10^{-9}M). It cannot therefore be determined whether the rat system would have shown an effect of MBP on testosterone production at 10^{-5}M MBP. The reduction of testosterone output when MBP was co-incubated with hCG but not with 22-R-CHO, suggests that MBP targets part of the testosterone biosynthesis cascade initiated by the binding of hCG to the LH receptor, but upstream of the point at which 22-R-CHO exerts its effect. This conclusion was comparable with the results of the parallel experiments using fetal rat testis explants.

It has been reported that rodents differ conspicuously from primates in their manner of metabolizing phthalates so the relevance of any differences must be clarified before rodent species can be understood as models for the effects of phthalates in humans (Albro et al., 1982). However, the findings from these present studies do indicate the possibility of a reproducible effect of MBP exposure on testosterone production in both fetal rats and fetal humans but further experiments are required to reconcile these findings.

But, it must be emphasised that as neither hCG nor MBP had a significant effect on basal testosterone production, it is not readily explained why the combination of these two compounds should reduce testosterone production consistently and at two concentrations of MBP, other than the possibility of these data being chance results, perhaps due to culture variability. One of the limitations of the present studies is the multiple-comparison situation:

only a small number of samples were available and each testis was divided into explants that underwent different treatments. Thus, the number of statistical tests performed was restricted and some may randomly reach statistical significance. It is possible that this phenomenon could account for the significant reduction in the level of testosterone produced when explants were co-incubated with 10^{-5}M MBP and hCG compared to hCG alone (section 5.3.3.3). However, this result was reproduced at two doses of MBP and was seen with 10^{-3}M MBP and hCG in the in vitro rat experiments and furthermore is consistent with current understanding of the mechanism of MBP toxicity.

Not all the adverse effects on rat fetal testis development induced by in vitro MBP exposure were successfully reproduced in fetal human testis explants incubated under identical conditions for the same duration. The inability of this system to reproduce all of the same endpoints as the in vitro studies reported in Chapter 4, is considered most likely to be the consequence of the species difference and not the method used. Such a difference has been reported before. Lin et al (1995) compared lipid synthesis in human and rat hepatocytes and reported that propionate inhibited cholesterol synthesis in 50% of the rat cells at 0.1mM/l but human hepatocytes required 10-20mM/l propionate to achieve an equal level of inhibition under identical primary culture conditions (Lin, 1995). This species-specific difference in sensitivity to metabolic agents could contribute to the difference in sensitivity to inhibition by Ketoconazole, seen with the human explants but not seen in the rat experiments.

It is difficult to compare the dose of a compound delivered to rodents experimentally to the dose of the same compound delivered to humans through uncontrolled low level environmental exposure. The ultimate aim of using animal models to investigate medical problems is to obtain findings that are predictive of the situation in humans. Second trimester human fetal testes (14-20w/40w) were available from legal abortuses, enabling parallel studies to those undertaken with rat fetal testis explants, to be undertaken with human fetal testes.

However, the lack of developmental parallels between these two species and the developmental stage of the samples available (second trimester fetal human testes vs. late gestation fetal rat testes) restricted the degree to which these aims could be achieved.

For example: the main cell types of the testis were at inconsistent phases of proliferation or maturity, germ cells were quiescent in the rat but proliferating in the human samples whereas the opposite was true of Sertoli cells, quiescent in the human yet proliferating in the rat. Also, the steroidogenic cells showed a disperse arrangement in the human samples compared to their distinct aggregation in the rat testes. However, both systems used testes explanted

around their peak of testosterone secretion, 12-16/40w in the human and e18-19.5 in the rat (El-Gehani et al., 1998; Voutilainen, 1992).

In conclusion, these data show that fetal human testes can be successfully cultured under the outlined conditions for up to 48h. The incorporation of the proliferation marker BrdU and the production of testosterone confirm that the explanted testes, dissected at 14-20/40w, were viable in this organotypic culture system.

6 Studies using short-term exposure of the fetal rat to phthalates

6.1 Introduction

The original in vivo studies reported in Chapter 3, aimed to investigate whether in utero exposure of male rats to 500mg DBP/kg/day from e13-e21.5 would induce a range of malformations in the reproductive tract that paralleled human TDS. The extent, to which this was achieved, is discussed at the end of Chapter 3. The inability to induce the same disorders or effects after exposure of fetal testis explants to the DBP metabolite MBP over a 48h period in vitro, raised the question of whether this was related to the age of the fetus at first exposure to MBP/DBP (i.e. e19.5) and/or the short (48h) duration of exposure (Chapter 4).

Hence, in vivo studies were set up to investigate whether it was feasible for 48h exposure, from e19.5-e20.5 (DBP 500 S), to induce any of the changes in testis morphology and testosterone production seen in the original studies involving exposure to DBP from e13.5-e20.5 (DBP 500 L).

6.2 Methods and Materials

The aim of these studies was to establish whether it was feasible for DBP to induce phenotypic changes to the fetal rat testis collected at e19.5, then maintained in vitro for 48h (Chapter 4). For the in vivo model, pregnant rats were treated on e19.5 and e20.5 only and the testes collected for study on e21.5. These were compared with age-matched animals subjected to a longer DBP treatment regime (e13.5 to e20.5) as reported in Chapter 3. Three additional treatments were used in these preliminary investigations, all administered on e19.5 and e20.5:

- DBP 500 S = 500mgDBP/kg/day (n=3)
- MBP 500 S = 500mgMBP/kg/day (n=1 (1 dam died prior to end of treatment)
- MBP 185 S = 185mgMBP/kg/day (n=2)

These were compared to previous studies with longer dosing regimes, daily from e13.5 to e20.5:

- Control = 1ml Corn oil/kg/day (n>4)
- DBP 500 L = 500mgDBP/kg/day (n>4)

There were no obvious differences between the testes exposed to vehicle from e13-e20.5 compared to e19-e20.5 (n=1), so control data were combined for statistical analysis. The focus for the investigations was the comparison of the two DBP treatment regimes. The MBP 500 S animal (n=1) was added to enable a comparison between effects induced by the metabolite as used in the in vitro studies and the parent compound (DBP) as used for the in vivo studies. Two dams were treated with a dose of 185mg MBP/kg bodyweight. This dose level was calculated to approximate the top dose of 1mM MBP used in the in vitro experiments. It has been determined that a fetus receives 0.12-0.15% of the administered maternal dose (Saillenfait et al., 1998).

Therefore, 0.12% = 1/833, so if the in vitro studies exposed the fetal testis to 1mM, the equivalent maternal dose would be 833mM. Given that the molecular weight of MBP = 222, 1mMol = 0.222g/l, 833mMol = 185g/l = 185mg/ml of vehicle, dosed at 1ml/kg = 185mg/kg.

Tissue was analysed using the same general methods as used in Chapter 3.

6.2.1 Statistical analysis

Due to the small sample size used for these studies, statistical analysis was not always possible. Specific computer software was used to assist in the data analysis, as previously described in Chapter 2 (GraphPad Prism v4.0b for Macintosh, GraphPad Software, San Diego, USA).

6.3 Results

Following in vivo treatment, fetal testes were recovered at e21.5; left testes were weighed, Bouin's fixed, sectioned and immunostained as required, whereas right testes were snap frozen until processed for protein or steroid level investigations. At e21.5, each major testis cell type was observed and analysed separately, namely Sertoli cells, gonocytes and Leydig cells. The peritubular myoid cells, that surround the developing testis cords, had not shown any obvious treatment related changes with the longer dosing regime so were not investigated.

6.3.1 Effect of fetal exposure to DBP on e21.5 testes

Testis weight was recorded in e21.5 rats and the mean testis weight per litter per treatment regime was calculated. DBP 500 L treatment induced a significant decrease in testis weight compared to Control treatment as reported previously and DBP 500 S induced a slight decrease from the control mean of 1.384mg to 1.345mg (Figure 6.1). The reason for this consistent decrease in weight was investigated at a cellular level.

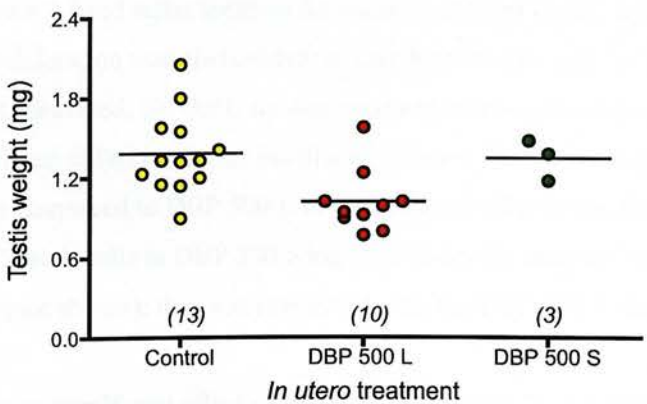

Figure 6.1: Mean testis weight per litter (mg) of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. Individual mean per litter (n) and mean per treatment (horizontal line) are plotted (n values are shown in parentheses).

6.3.1.1 Sertoli cells

Immunostaining of testis sections for the Sertoli cell nuclear marker WT-1, showed Sertoli cell nuclei at the periphery of the testis cords in all samples at low magnification (not shown). Review of the slides at higher magnification, revealed WT-1 stained cells in the interstitium of treated testes (Figure 6.2). This abnormal staining pattern was consistent in testes following either the long-term or the short-term exposure regime and was absent from vehicle-exposed testes.

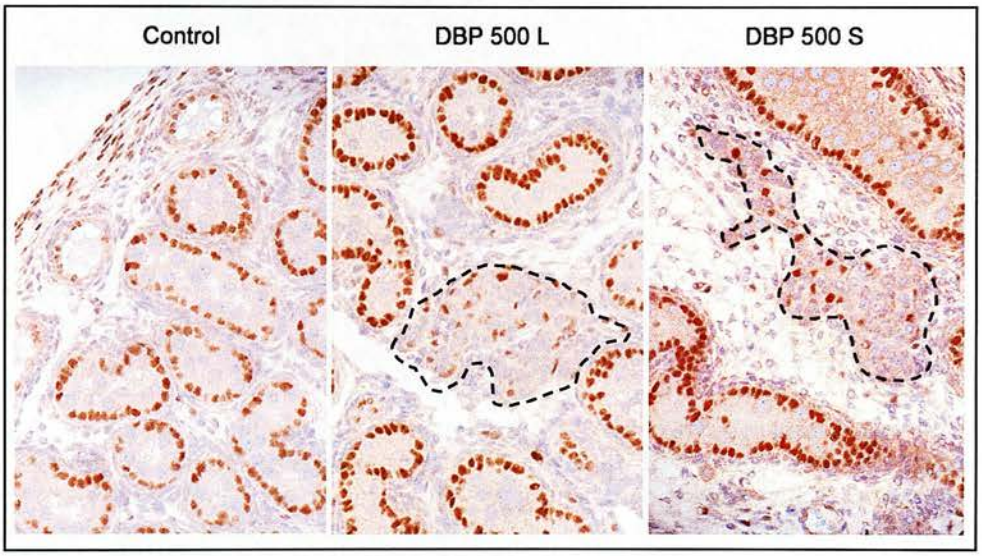

Figure 6.2: WT-1 immunostained sections of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. Note the positioning of stained Sertoli cell nuclei, at the periphery of the developing cords. Additional staining was seen within abnormal "dysgenetic" interstitial areas of both DBP exposed testes (dashed outlines). The panels show a cross section through a whole testis photographed using a x40 objective.

The effect of in utero DBP exposure on the rate of Sertoli cell proliferation was measured by immunostaining Bouin's fixed testis sections for incorporation of BrdU, injected into the mother 1.5h prior to kill. Proliferation was also evident in non-Sertoli cells (e.g. interstitial cells, Figure 6.3) but this was not measured. No BrdU uptake was seen in any gonocytes (Figure 6.3, inset). There was no significant difference in the proliferation index for Sertoli cells in the DBP 500 S exposed testes when compared to DBP 500 L or the respective Controls (Figure 6.4). Immunostaining of Sertoli cells in DBP 500 S exposed testes for markers of apoptosis (TUNEL) showed no staining (not shown); this was consistent with the DBP 500 L data as detailed in Chapter 3.

Given that there was no significant effect of treatment on the levels of inhibin-B production seen in either the original in vivo or the in vitro studies, inhibin-B levels were not compared between the two in vivo regimes.

Overall, regarding Sertoli cell effects, the DBP 500 S exposure regime showed a similarly low level of adverse effects on testis development, as did the DBP 500 L regime.

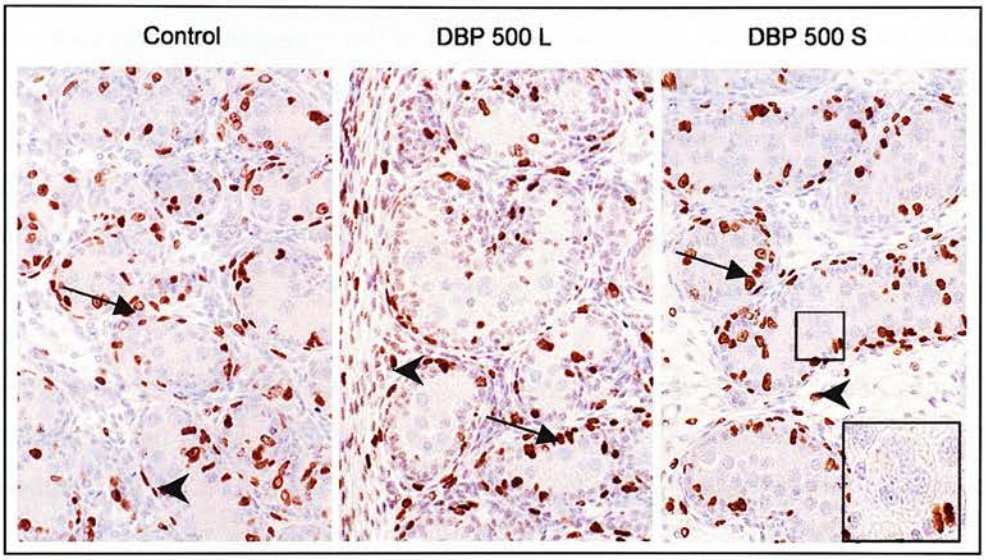

Figure 6.3: BrdU immunostained sections of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. BrdU administered 1.5h prior to testis collection, was incorporated by intratubular Sertoli cells (arrow) as well as interstitial cells (arrow head) but not intratubular gonocytes (inset). There was no obvious effect of treatment on the level of BrdU staining. The panels were photographed using a x40 objective, with an enlarged inset.

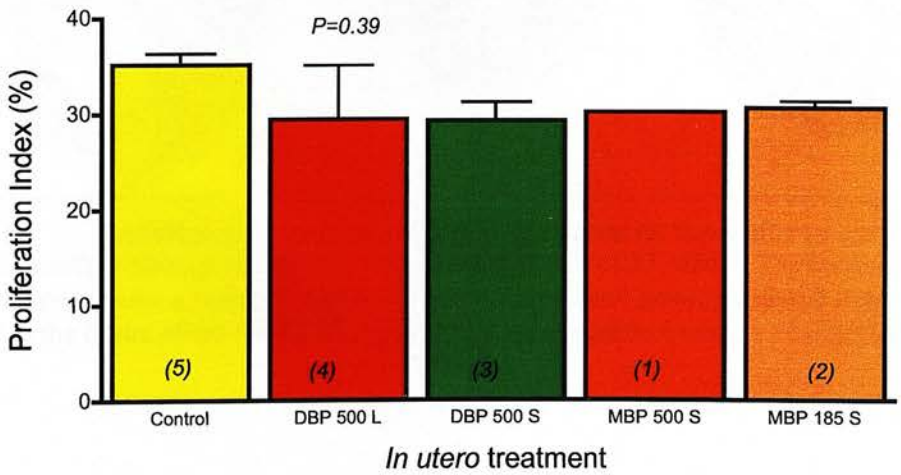

Figure 6.4: Proliferation Index (%) of Sertoli cells in e21.5 fetal rat testes after e13.5-20.5 or e19.5-e20.5 in utero exposure to: vehicle (combined data = Control) or either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day or e19.5-20.5 in utero exposure to 500 or 185 mgMBP/kg/day (MBP 500 S and MBP 185 S respectively). Values are litter means where n=1-2 and litter means ± S.E.M. where n>2 (n values are shown in parentheses). There was no significant effect of any treatment regime on Sertoli cell PI.

6.3.1.2 Gonocytes

Testis sections were immunostained for AMH, a protein expressed in the Sertoli cell cytoplasm that surrounds the fetal gonocytes. Low magnification reviews of the AMH staining demonstrated that the number of cords present per testis after DBP exposure was altered (Figure 6.5). This difference was quantified and revealed a significant reduction in the number of cords per section following even short-term DBP exposure, compared to the control sections (Figure 6.6). Whether this reduction was the result of fewer cords or reduced coiling of the same number of cords could not be deduced by these studies. The effect of treatment on mean cord diameter was not quantified. It should be noted that the plane of section analysed per sample and per treatment were comparable.

Further review of the sections showed normal AMH negative areas between the developing cords in control and treated sections. DBP 500 L treated sections also showed areas between cords with AMH positive cells but these were not observed in the DBP 500 S treated sections (not shown).

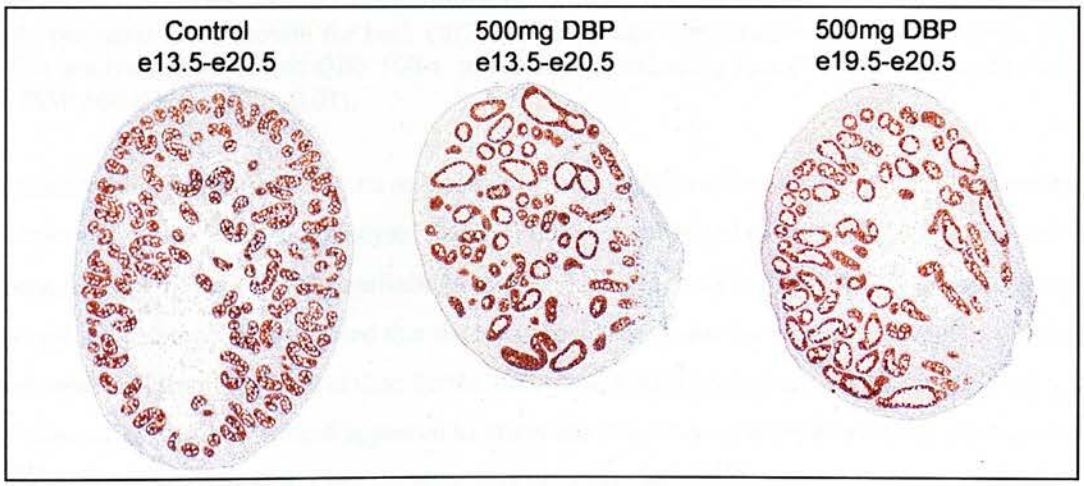

Figure 6.5: AMH immunostained sections of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. The sections from DBP-exposed animals show a reduced number of cords with altered gonocyte distribution (abnormally clustered in the centre of the cords). The panels were photographed using a x4 objective.

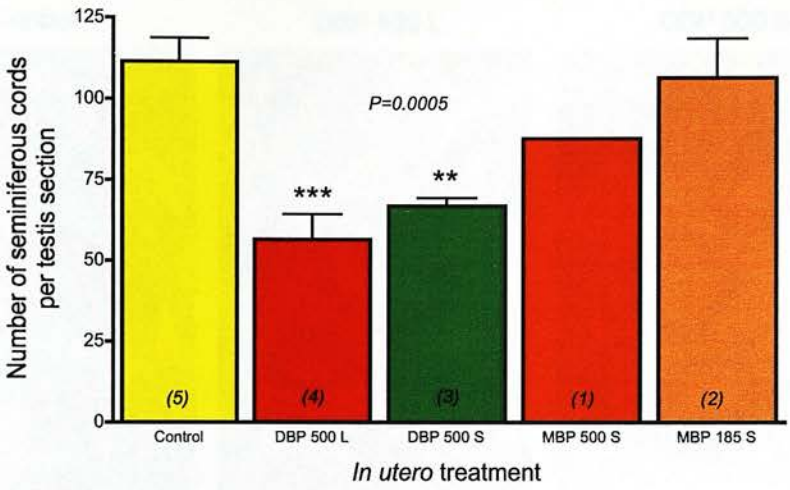

Figure 6.6: Number of seminiferous cords per cross section of e21.5 fetal rat testes after e13.5-20.5 or e19.5-e20.5 in utero exposure to: vehicle (combined data = Control) or either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day or e19.5-20.5 in utero exposure to 500 or 185 mgMBP/kg/day (MBP 500 S and MBP 185 S respectively). Values are liter means where n=1-2 and litter means ± S.E.M. where n>2 (n values are shown in parentheses). ANOVA showed a significant (P=0.0005) decrease in the number of seminiferous cords per testis cross section for both DBP treated groups compared to the control testes, though further analysis showed that DBP 500 L testes had significantly less (***P<0.001) cords than did the DBP 500 S testes (**P<0.01).

Examination of AMH stained testis sections at higher magnification (>x40) revealed the presence of abnormal multinucleated gonocytes (MNGs) in testes subjected to the DBP 500 S exposure regime (Figure 6.7). This was consistent with the observations of the DBP 500 L exposed testes reported in Chapter 3. It was noted that the mean incidence value for MNGs was higher with the short-term DBP regime (53.6%) than for the long-term DBP exposed testes (37.1%) (Figure 6.8). The dose of MBP administered appeared to affect the incidence of MNGs inversely, though this was not investigated further. Overall, regarding gonocyte effects, the short-term phthalate exposure regime showed at least an equivalent level of adverse change as did the long-term regime, if not slightly more severe.

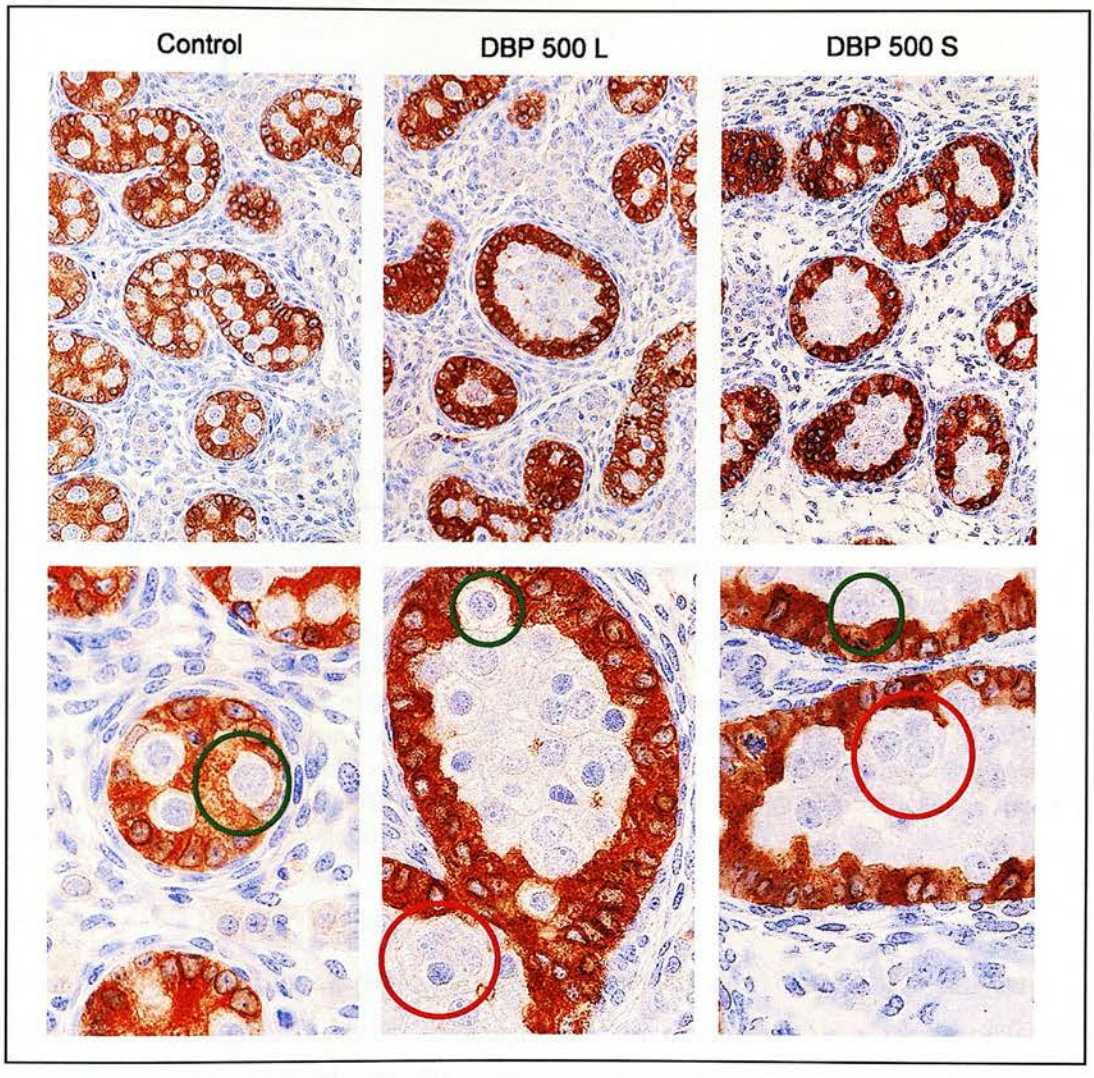

Figure 6.7: AMH immunostained sections of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. The Control sections show normal mononucleated gonocytes (green circles) dispersed across even-sized cords compared to the distended cords in the sections from DBP-exposed animals and their abnormal gonocyte positioning. Abnormal multi-nucleated gonocytes (red circles) were present in the testis cords following both DBP exposure regimes. The top panels were photographed using a x40 objective and the lower panels were photographed using a x100 objective.

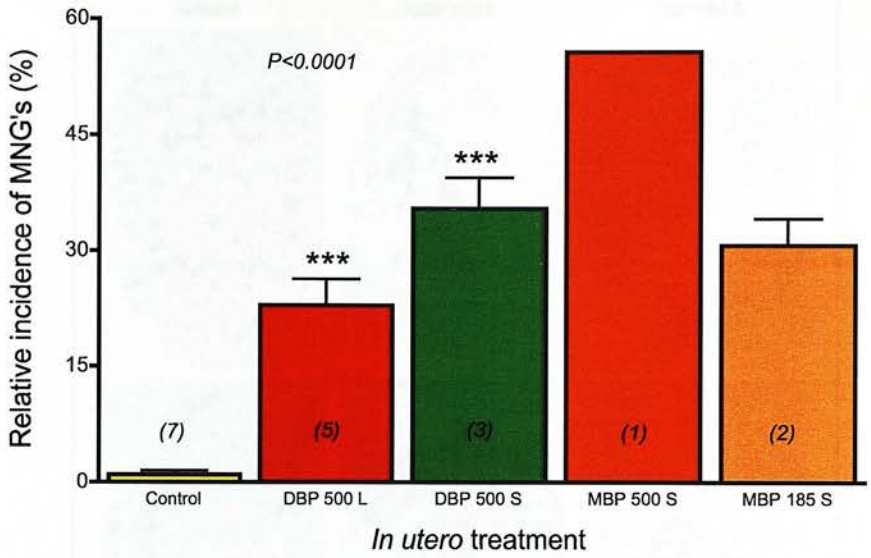

Figure 6.8: Incidence (%) of multinucleated gonocytes (MNGs) in e21.5 fetal rat testes after e13.5-20.5 or e19.5-e20.5 in utero exposure to: vehicle (combined data = Control) or either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day or e19.5-20.5 in utero exposure to 500 or 185mgMBP/kg/day (MBP 500 S and MBP 185 S respectively). Values are litter means where n=1-2 and means ± S.E.M. where n>2 (n values are shown in parentheses). All DBP exposed groups showed a highly significant increase in the frequency of MNGs (*** P<0.001).

6.3.1.3 Leydig cells

Normal untreated/ uncultured fetal testes aged e21.5 contain small clusters of Leydig cells, disseminated throughout the testis. Normally, each cluster occupies <5% of the total Leydig cell area per testis section, resulting in >20 clusters per section. Occasionally, control Leydig cell clusters occupy between 5-15% of the total Leydig cell area per section but clusters >15% were rarely seen in testis sections from untreated animals. Clusters were categorised by these percentage areas and defined as either small (<5%), medium (5-15%) or large (>15%). The effect of DBP exposure on the distribution of Leydig cell clusters was quantified using computer assisted stereological techniques, detailed in Chapter 2.

Three sections per sample were assessed in three pups per litter and the mean values compared between samples (Figure 6.9). The percentage of the total testis section area that was occupied by Leydig cells and for each of the cluster categories was analysed (Figure 6.10).

The distribution of the Leydig cells showed a trend away from small clusters with phthalate exposure, compared to vehicle controls, with the largest shift seen in testes from animals exposed to DBP under the DBP 500 L regime.

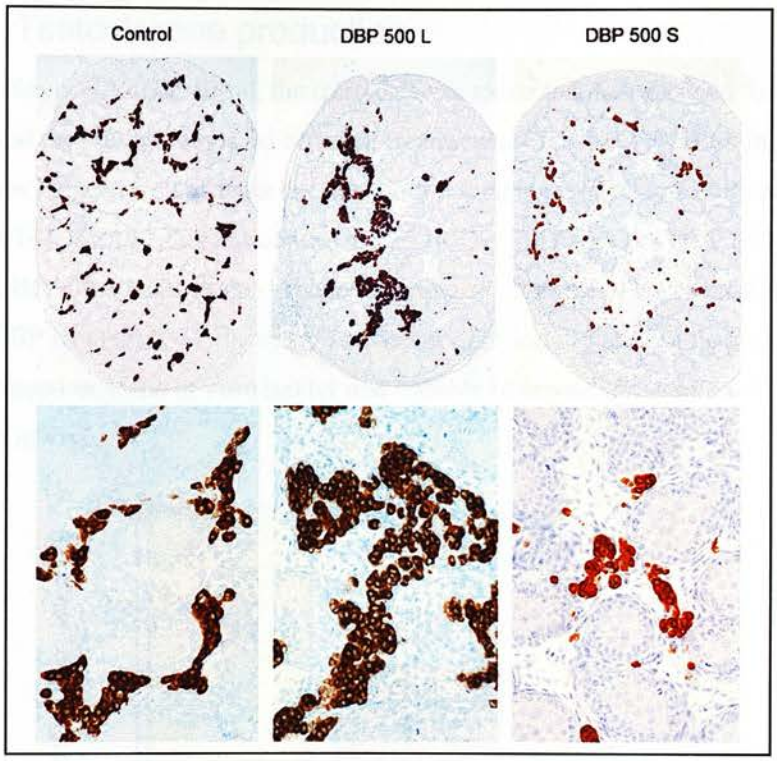

Figure 6.9: 3β-HSD immunostained sections of e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 or e19.5- e20.5. Note the markedly larger Leydig cell clusters in the DBP 500 L sections, compared to the control sections. The top panels were photographed using a x10 objective and the lower panels were photographed using a x40 objective.

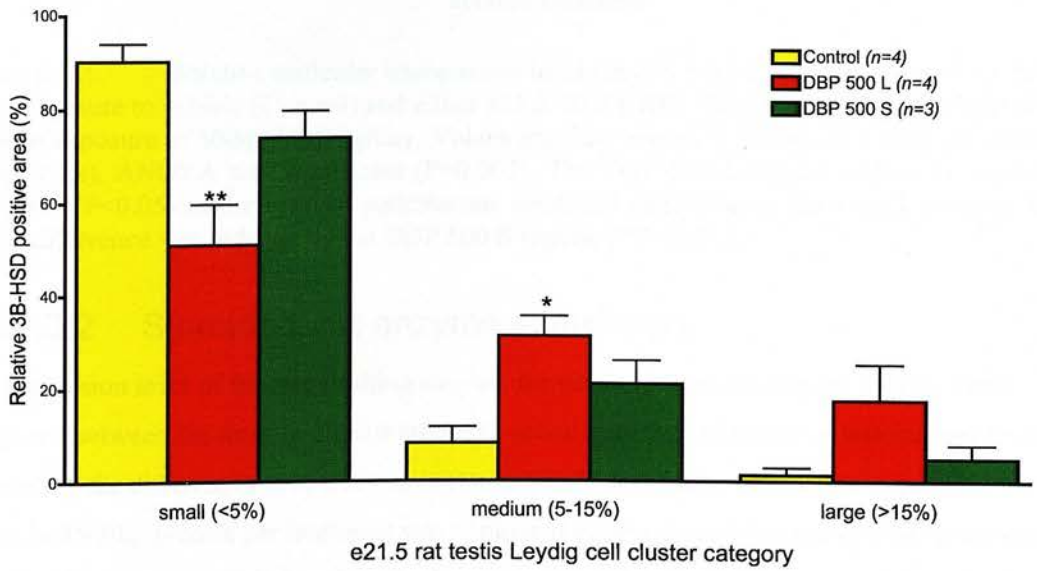

Figure 6.10: Leydig cell cluster size index (%) of 3βHSD immunopositive stained areas in e21.5 fetal rat testes after in utero exposure to vehicle (control) or 500mgDBP/kg/day from e13.5-e20.5 (L) or e19.5- e20.5 (S). Values are litter means ± S.E.M. (n values are shown in parentheses). Both DBP exposure regimes produced a decrease in the percentage of Leydig cells in small clusters and a shift towards an increase in medium and large clusters, though this change was only significant for the e13.5-e20.5 (L) exposed testes (** $P<0.01$, * $P<0.05$).

6.3.1.3.1 Testosterone production

One testis per litter was homogenised, the steroids were extracted then assayed for testosterone levels by RIA and the values compared between treatments (Figure 6.11). Both in vivo DBP exposure regimes induced a significant decrease in the total level of testosterone per testis (ANOVA P=0.014), from 17.8ng per control testis (100%) to 10.4ng per DBP 500 L testis (59%) and 5.9ng per DBP 500 S testis (33%). This result suggests that the 48h exposure of testis explants in vitro to the DBP metabolite MBP, was of sufficient duration to induce a decrease in basal testosterone production, if the in vitro model was capable of reproducing phthalate induced changes as seen in vivo.

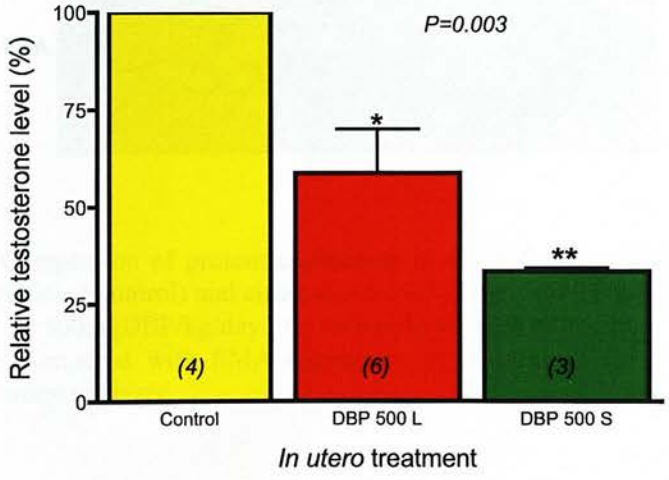

Figure 6.11: Relative testicular testosterone levels in e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. Values are litter means ± S.E.M. (n values are shown in parentheses). ANOVA was significant (P=0.003). The DBP 500 L regime induced a significant decrease (*P<0.05) in the level of testosterone produced compared to the vehicle control, but a bigger difference was induced by the DBP 500 S regime (**P<0.01).

6.3.1.3.2 Steroidogenic enzyme expression

The expression level of the rate-limiting enzyme for testicular steroidogenesis ($P450_{scc}$) was compared between the three treatment groups to investigate the difference in testosterone level induced by the different in vivo treatments (Omura and Morohashi, 1995). The expression level of testicular $P450_{scc}$ protein per treatment was compared against that of the non-steroidogenic protein Smooth Muscle Actin (SMA) as described previously (section 3.3.1.3). Expression levels of $P450_{scc}$ and SMA were measured and compared per treatment following Western Blot analysis of extracts of total testis protein (Figure 6.12). The expression levels were quantified using computer assisted image analysis (Image Quant) and relative expression data for $P450_{scc}$ was subject to statistical analysis (Figure 6.13).

DBP treatment showed a highly significant (P=0.0014) decrease in the level of $P450_{scc}$ expression relative to SMA expression when compared to untreated testes. This study demonstrated that the rate-limiting step of steroidogenesis was compromised by both DBP exposure regimes.

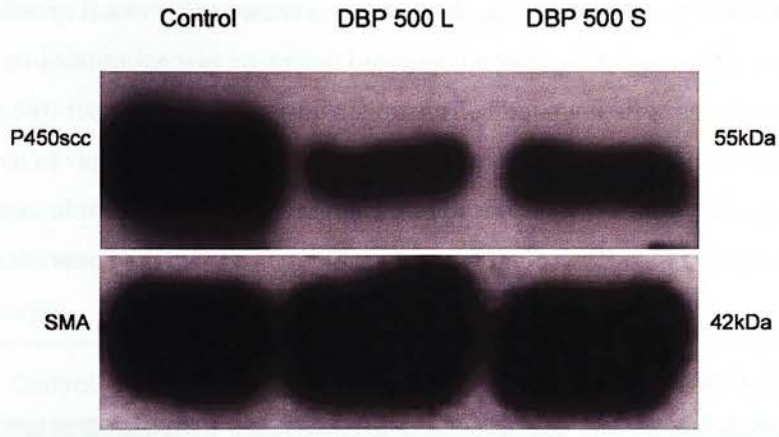

Figure 6.12: Comparison of protein expression in e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. An example of a Western blot showing the level of $P450_{scc}$ expression compared with SMA expression is illustrated. This was quantified using computer assisted image analysis.

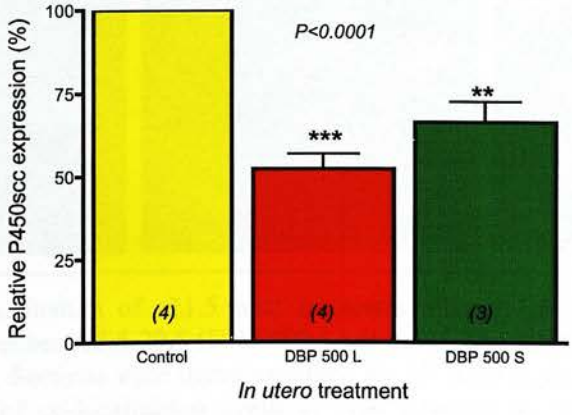

Figure 6.13: Relative $P450_{scc}$ protein expression in e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. Values are litter means ± S.E.M. (n values are shown in parentheses). ANOVA was highly significant (P<0.0001). The DBP 500 L regime, induced a significant decrease (***P<0.001) in the level of testosterone produced compared to the vehicle control, with a significant but smaller difference induced by the DBP 500 S regime (**P<0.01).

An additional experiment compared the expression pattern of $P450_{scc}$ in the Leydig cells with that of another steroidogenic enzyme, namely 3β-HSD. This investigated whether $P450_{scc}$ expression levels did vary across the testis or whether any variations were localised. This experiment was facilitated by the use of fluorescent co-localisation of immunostaining of $P450_{scc}$ and 3β-HSD protein expression in Bouin's fixed testis sections, as described previously (Section 3.3.1.3). The distribution of co-localisation was compared between the treatments against the vehicle control testis (Figure 6.14). Expression levels were not quantified but qualitative assessment suggested that there was no obvious treatment related change in the distribution of the two proteins between the testes of either of the DBP-exposure regimes and/or the vehicle control. The reduction in $P450_{scc}$ expression seen with the Western blot analysis (Figure 6.12) could not be reproduced using this technique.

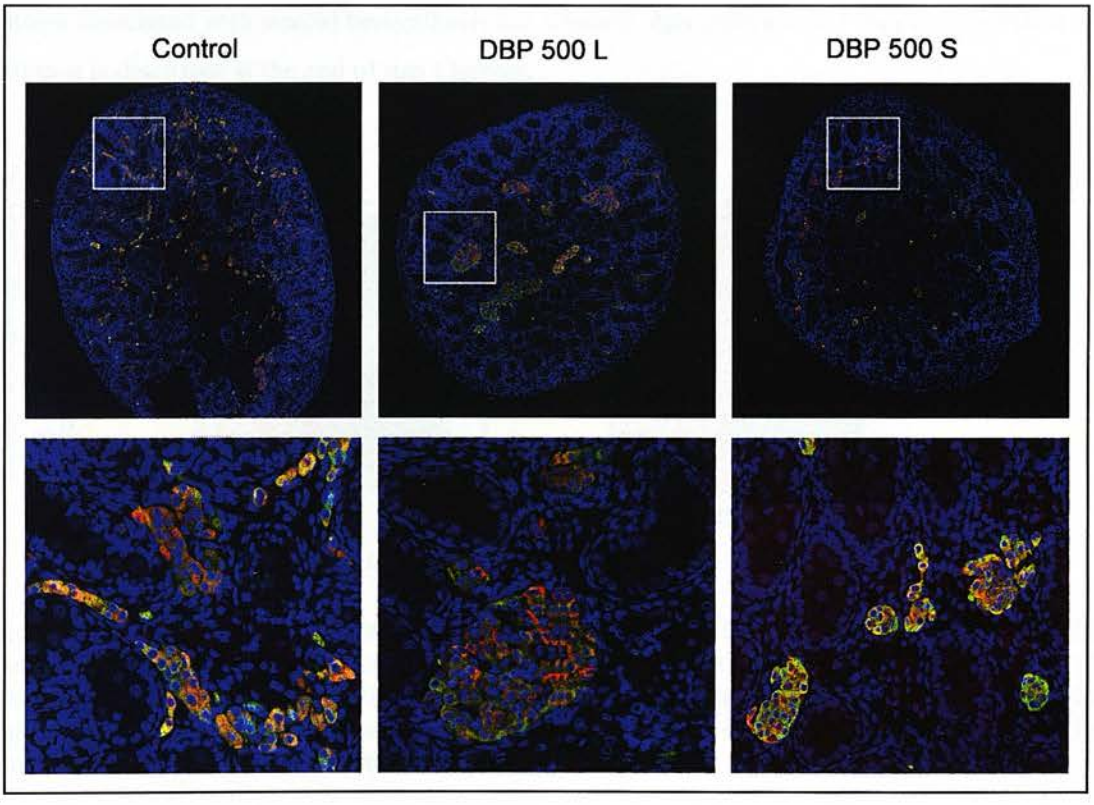

Figure 6.14: Examination of e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. Sections were immunostained for 3β-HSD (red) and $P450_{scc}$ (green) to see whether the pattern of co-localisation (yellow) was affected by treatment. Blue = nuclear counterstain. An enlarged image of the region in the white box can be seen below the corresponding image, taken at x40 magnification.

Additional Western blot analyses were performed, using samples from one animal but in duplicate. These trial studies quantified blots of: 3β-HSD, Inhibin-α expression in testes and $P450_{scc}$ expression in adrenals of the same animals. The relative levels of expression were

determined, compared to testicular SMA as previously, though small groups sizes (n=1, in duplicate) prohibited any statistical analyses (Figures 6.15 to 6.17). These preliminary experiments revealed that there was no obvious effect of the treatment regimes on the level of $P450_{scc}$ expression in the adrenal glands, unlike in the testes (Figure 6.12) and that 3β-HSD expression in the tests was not obviously affected by the treatments (Figure 6.15), unlike testicular $P450_{scc}$. Finally, a "quick look" at testicular inhibin-α hinted that its two isoforms detected by the antibody used might be differentially affected by in utero exposure to 500mgDBP/kg/day over both exposure regimes (Figures 6.16 and 6.17). Fetal testis inhibin-α has been shown previously to produce two bands under reducing conditions, reported as measuring approximately 47 and 50kDa (Noguchi et al., 1997).

These data support the idea that the effect of DBP is specific to the testis and that not all the proteins associated with steroid biosynthesis are affected. Any relevance of the possible effect on inhibin-α is discussed at the end of this Chapter.

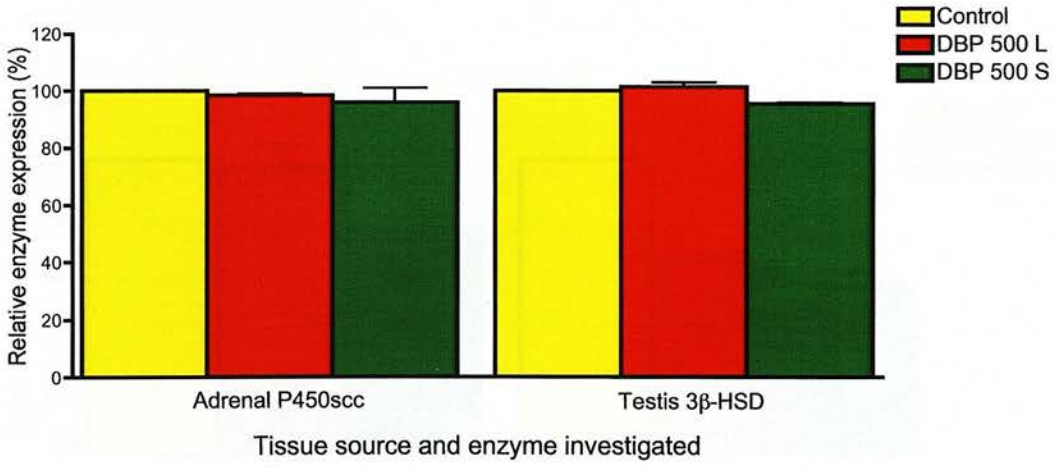

Figure 6.15: Relative protein expression in e21.5 fetal rat testes or adrenals after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. Values are sample means ± SD's (n =2). No statistical analyses were performed. The data suggest there was no obvious effect of either treatment regime on adrenal levels of $P450_{scc}$ or on testis levels of 3β-HSD.

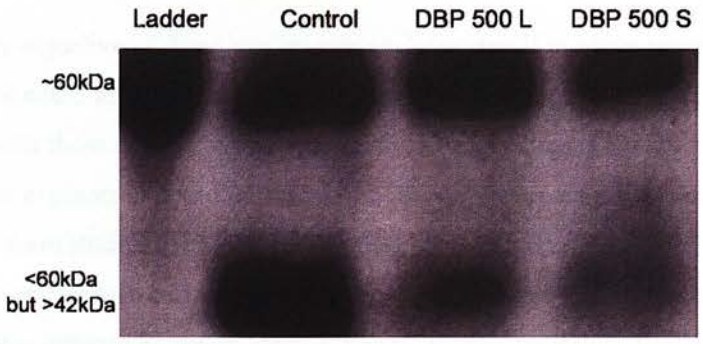

Figure 6.16: Comparison of protein expression in e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. An example of a Western blot, showing the levels of expression of two inhibin-α related proteins of differing molecular mass is illustrated. This was quantified using computer assisted image analysis and expressed relative to SMA expression.

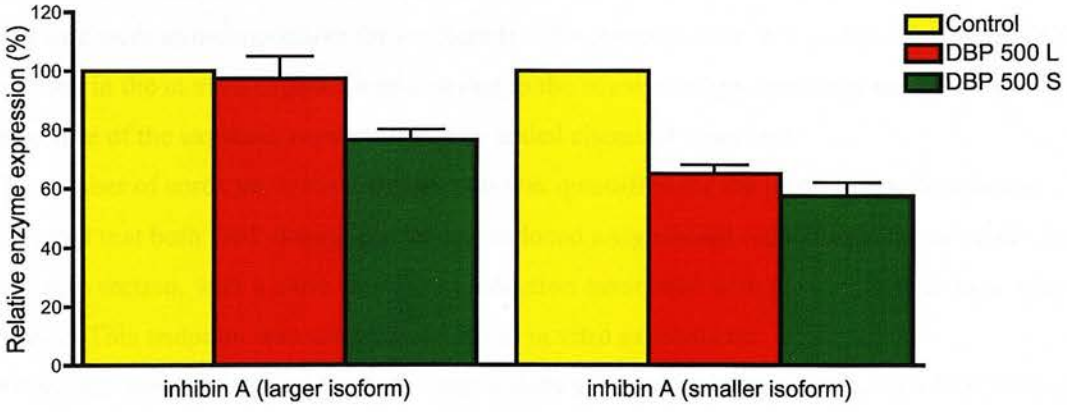

Figure 6.17: Relative protein expression in e21.5 fetal rat testes after e13.5-20.5 in utero exposure to vehicle (Control) and either e13.5-20.5 (DBP 500 L) or e19.5-20.5 (DBP 500 S) in utero exposure to 500mgDBP/kg/day. Values are sample means ± SD's (n =2). No statistical analyses were performed. The data suggest there might be an effect of both treatment regimes on testis levels of two inhibin-α related proteins, though it is unclear why there may be a greater effect with the shorted treatment regime.

Overall, regarding Leydig cell effects, the DBP 500 S exposure regime showed a similar level of adverse effects on testis development as the DBP 500 L regime, with reduced testosterone levels and $P450_{scc}$ enzyme expression. Further studies would be needed to confirm the lack of effect on adrenal $P450_{scc}$ or testicular 3β-HSD as well as to investigate the possible significance of an effect on the expression of inhibin-α.

6.4 Discussion

The primary objective of these studies was to determine if in utero exposure to 500mgDBP/kg/day on e19.5 and e20.5 affected normal testis morphology and growth by e21.5. Any changes were compared with those seen in testes exposed for a longer time (e13-e20.5, Chapter 3) or withthose seen in testis explants exposed in vitro to 10^{-3}M MBP over a similar duration (48h, Chapter 4). The longer–term studies (e13-e20.5, DBP 500 L), induced an array of changes in the cellular architecture and protein expression of the fetal testis as reported in Chapter 3, These are compared to the changes induced by the reduced exposure studies (e19+e20.5, DBP 500 S) reported in this Chapter.

Both regimes induced a decrease in testis weight at e21.5, though this was not significant for the DBP 500 S litters. Given that the weight of a testis is affected by the number of cells it contains, the level of proliferation was measured for the Sertoli cells (Orth, 1984). Neither regime was able to induce a significant effect on the proliferation index of Sertoli cells. This endpoint had been unaffected in the in vitro studies also.

Both in utero regimes induced a change in testis architecture with areas between cords containing cells that were immunopositive for the Sertoli cell nuclear protein WT-1. This change could not be assessed in the in vitro exposed explants due to the consistent appearance of necrotic regions in the centre of the explants, regardless of any added chemical treatment.

The number of cords per testis cross-section was quantified for the in vivo experiments and indicated that both DBP-dosing procedures induced a significant reduction in the number of cords per cross section, with a more significant reduction associated with the longer-term exposure method. This endpoint was not assessed in the in vitro experiments.

Within the cords, the gonocytes were dramatically affected by both of the in vivo DBP treatment routines. Both procedures induced the appearance of abnormal multinucleated gonocytes (MNGs). However, there was a greater incidence of cords containing MNGs in the DBP 500 S (mean = 36%) testes than in the DBP 500 L (mean = 23%) testes examined, compared to the control testes (1%). Why the reduced exposure scheme should have had a more severe effect, is unclear. This endpoint was assessed also in the in vitro-exposed explants but there was such a high incidence of MNGs in the untreated explants (>15%) that there was no significant effect seen following phthalate exposure (>20%).

Examination of the Leydig cells saw a significant change in their distribution pattern following DBP 500 L exposure, with a shift away from small clusters that each accounted for <5% of the total area of Leydig cells per testis, towards clusters that measured >5% (either 5-15% or >15%). This pattern was also seen in DBP 500 S exposed testes though the shift was less pronounced and was not statistically significant. A similar pattern was revealed in the in vitro exposed explants

though this was not significant either. The number of Leydig cells was unaffected by the DBP 500 L regimen, so was not quantified following either the DBP 500 S or in vitro exposure routines. The level of testicular testosterone was significantly affected by both in utero DBP treatment regimes, though there was a greater reduction in animals treated according to the DBP 500 S scheme (33% of control) than the DBP 500 L regime (59% of control). However, the in vitro system had been unable to demonstrate any adverse effect of 10^{-3}M MBP on basal testosterone production.

The level of expression of the steroidogenic enzyme $P450_{scc}$ was quantified in testes exposed to DBP in vivo. When compared against the relative expression in control testes, both DBP treatment regimens significantly reduced the levels of $P450_{scc}$, with the greatest reduction associated with the DBP 500 L treatment. This endpoint was not assessed for the in vitro exposed explants, as there was no effect of phthalate exposure on basal testosterone production.

Overall, the DBP 500 S regime induced the same adverse effects as did the DBP 500 L dosing routine, and to a similar degree. These results confirm that is possible to induce adverse effects on fetal rat testis development with just 48h of phthalate exposure. Of the eight endpoints that were compared, the two in utero treatments showed similar effects in all of them, with the DBP 500 S regime having a more significant effect on the number of cords with MNGs and reducing testicular testosterone to a greater extent than did the DBP 500 L regime. The in vitro system was only able to show a shift towards the DBP-associated profile of Leydig cell distribution, with no significant effect on any other parameter measured.

Taken together, these data do not support the case for the use of the in vitro system, as utilised in Chapter 4, to make a significant contribution to exploring the cellular and molecular mechanisms behind the developmental toxicity induced by in utero DBP- exposure. This conclusion also mitigates against the in vitro system being used to compare the effects of phthalate exposure on explants of the fetal rat and human testis. This is unfortunate as this approach had the potential to enable the direct exposure of human tissue to controlled levels of phthalates and accurately track any changes induced, thus making an invaluable contribution to the assessment of risk from these chemicals to human health.

These data support the use of the shorter in utero DBP- exposure regime to help pinpoint the stages of development most vulnerable to DBP toxicity and to further our understanding of the normal development of the male reproductive tract. This in turn furthers our understanding of the risk that endocrine disrupting chemicals pose to human reproductive health. It should be noted that though there are broad similarities in the basic mechanisms of reproductive tract development in rodents and primates, there are differences. For this reason, it is critical to determine the details

of a chemical's mechanism of action before extrapolating effects observed in studies using rats to potential effects in man (Gray et al., 1989).

To this end, the DBP 500 S studies reinforced the dramatic effect that DBP 500 L exposure had on fetal testosterone production as shown in Chapter 3. Considering the profile of effects induced by the in vivo administration of DBP, various hypotheses have been tested that examine possible mechanisms behind DBP stimulated toxicity, hence possibly associated with the human condition TDS.

Lesions in the male reproductive tract have been noted from as early as e17.5, in the Leydig cells, when following the DBP 500 L dosing regime (Mahood et al., 2005). By determining the sequence of the lesion formation from e16.5 to adulthood and comparing how lesions change with age, it has been proposed that this would indicate the primary sites of DBP-induced pathological changes and indicate possible mechanisms behind the cellular and molecular pathogenesis (Barlow and Foster, 2003).

In the fetal testis, Barlow et al (2003) reported an increase in the incidence of three particular pathological lesions over e17.5 to e21.5 following in utero DBP-exposure, all of which were reproduced in the present studies, those reported in Chapter 3 and published by this laboratory (Mahood et al., 2005). The reported lesions are:

- larger but fewer Leydig cell clusters
- abnormally nucleated gonocytes with >2 nucleii
- larger but fewer seminiferous cords

Though the effects of the DBP 500 S regime on adult fertility has yet to be determined, by taking into consideration those effects associated with the DBP 500 L studies and published data, the postnatal DBP toxicity profile can be outlined. Following DBP-exposed animals through their postnatal development, Barlow et al (2003) reported all three of the fetal testis lesions present on postnatal d3 and d7. By d16, the abnormal gonocytes were gone but, a decreased number of spermatocytes was observed in DBP-exposed testes compared to untreated testes. By d21, the Leydig cell aggregates were no longer evident (Barlow and Foster, 2003). Mahood et al (2005) propose that the inter-tubular "Leydig cell" aggregates are not exclusively Leydig cells but are areas of intermingled cells including ectopic Sertoli cells and by d4 even show presumptive peritubular myoid cells and misshapen cord formation.

Curiously, at postnatal d3, 100% of the animals examined by Barlow et al (2003) had at least one testis lesion but by d7 this had fallen to 96% and by d21 this had reduced to just 85%. By d70, only 38% of the young adult DBP –exposed males had Leydig cell hyperplasia, but 100% of DBP-exposed litters had given rise to testes in which >75% of the tubules showed adversely affected spermatogenesis, as well as obvious lesions in their accessory sex glands and other

androgen-dependent tissues, such as hypospadias (Barlow and Foster, 2003). Additionally, this paper reported the effects of DBP exposure on the epididymides but concluded that these effects were likely to be secondary to the direct effect of DBP on the testes such as the decreased levels of testicular testosterone.

These studies were followed up in 2004 with a detailed look at the testis lesions in adult rats following in utero exposure to DBP (Barlow et al., 2004). Between 6-18 months old, the incidence of unilateral testicular lesions had risen from 36-75%, with little change for bilateral lesions increasing from 82-88% of DBP-exposed litters affected. Together with the present studies, these data reinforce the ability of DBP to instil irreversible changes in the adult testis following in utero exposure, akin to the TDS hypothesis (Skakkebaek et al., 2001).

However, the diverse cell populations and the dramatic levels of change in gene expression that are associated with normal fetal testicular development and the divergent phthalate metabolic pathways in primates and rodents, mean there is still uncertainty about the specific target of this chemical and its class (Akingbemi et al., 2004; Shultz et al., 2001).

Recent studies of the profile of changes resulting from in utero phthalate exposure, suggest the toxic effects are consistent with multiple endocrine disturbances (Barlow et al., 2003; Mahood et al., 2005; Thompson et al., 2004). DBP has been hypothesised to instigate its primary effect as a general reduction in the expression of testicular steroid biosynthesis related proteins and this is likely to be via LH independent mechanisms. This has been deduced as initiation of fetal rat testis testosterone biosynthesis, from e16.5, precedes the detection of LH in serum at e19.5 to stimulate the Leydig cells (El-Gehani et al., 1998). Work in this laboratory with DBP exposed testes has shown that Leydig cells are affected by DBP from e17.5, prior to LH stimulation (Mahood et al., 2005). However, it may be that DBP toxicity also manifests itself via LH dependent mechanisms, as LH-receptor levels have been shown to be down regulated by e21.5 (Shultz et al., 2001). Additionally the data presented in this Chapter, show that testicular testosterone levels are significantly reduced after exposure to DBP over just e19.5- e21.5 when serum LH is predicted to be influencing testicular testosterone production.

Mahood et al (2005) went on to point out that the testis specific effects of DBP are perhaps Leydig cell mediated. Yet even here, the effects within the Leydig cells are not general as the expression of certain enzymes (e.g. 3β-HSD) are unaffected by either DBP treatment regime (Mahood et al., 2005). This conclusion is consistent with the preliminary data reported in this Chapter where expression of the $P450_{scc}$ protein in DBP-exposed adrenal glands was not obviously reduced and neither was testicular 3β-HSD following either DBP exposure regime, compared to the significantly reduced level of testicular $P450_{scc}$ protein expression seen with both treatments. The lack of effect of these DBP-exposure regimes on the expression level of 3β-HSD protein seen in

these studies contrasts with significant decreases in 3β-HSD mRNA levels reported by other laboratories (Barlow et al., 2003; Shultz et al., 2001). However, Barlow et al (2003) also reported a disparity between their quantified, reduced mRNA and protein expression revealed by immunostaining (e.g. SCF data) and failed to support their significant reduction in 3β-HSD gene expression ($P<0.05$) with any data regarding 3β-HSD protein expression. Therefore, their conclusion that 3β-HSD protein expression must also be reduced in e19.5 testes following in utero DBP-exposure must be considered carefully. Additionally, Thompson et al (2003) showed that when DBP-exposed fetal testes were incubated with the steroidogenic intermediates normally catalysed by 3β-HSD (e.g. pregnenolone), then testosterone levels increased. Admittedly, control levels of the 3β-HSD catalysis products were not fully restored, but this data suggests that the expression level of 3β-HSD protein was high enough to support steroidogenesis despite reduced gene expression (Barlow et al., 2003).

However, none of these studies reported any change in the level of the gene or protein expression for inhibin-α as hinted at in the final experiments reported in this Chapter. Micro-array analysis of mRNA from e19.5 rat testes, after exposure to 500mgDBP/kg/day or vehicle from e13.5, showed a highly significant ($P<0.01$) decrease (-1.63 fold change) in inhibin-α. This data was generated through collaboration between this laboratory and CXR Biosciences[1], where the micro-arrays were performed and analysed. Data was only generated for e15.5, e17.5 and e19.5 testes treated according to this laboratory's DBP 500 L regime from e13.5.

Inhibins are heterodimeric glycopoteins involved in the regulation of a number of diverse physiological functions, including regulation of endocrine hormone production (e.g. FSH) and possibly germ cell development (Majdic et al., 1997). The α-subunit dimerises with any of the β-subunits (A, B or C in the human, plus E in the mouse, plus D in Xenopus), though in mammals, only the β_B subunit is associated with male reproductive function (Illingworth et al., 1996). It might be considered that any treatment might be expected to affect both dimers equally (α and β_B). However, in Chapter 3 there was no significant difference in the levels of intra-testicular inhibin β_B measured by ELISA in e21.5 testes treated with 500mgDBP/kg/day or vehicle from e13.5, while preliminary western blot analysis data presented in this Chapter, suggest the possibility of a change in expression of inhibin-α protein in e21.5 testes following the same exposure regimen. It has been reported that fetal testes express inactive forms of inhibin-α related proteins (molecular weights >40kDa) whereas the dimeric bioactive form (<30kDa) is not present until birth (Meachem et al., 2001; Noguchi et al., 1997). This is consistent with the immunoblots presented here, which showed the presence of two proteins of between 40-60kDa but no staining

[1] CXR Biosciences Ltd., James Lindsay Place, Dundee DD1 5JJ.

at 30kDa (not shown). It should be noted that small numbers of samples were assessed in all studies and that this effect of phthalates on inhibin-α has not been supported in the literature, but given the wide range of influence that both DBP and inhibins have on male reproductive health, the possibility that DBP may affect the maturation of inhibin-α from larger precursor molecules, may warrant further investigation.

Overall, these studies have extended our knowledge of the susceptibility of the fetal rat testis to developmental disruption by acute exposure to high doses of DBP. Exposure of 500mgDBP/kg/day is an unlikely exposure level for humans but the purpose of this model was to produce a high incidence of the adverse DBP-induced phenotype, without inducing maternal toxicity, thereby allowing changes in protein expression associated with the morphological changes to be detected (Barlow et al., 2003). Much further work is still needed to explore the cellular and molecular mechanisms behind the developmental toxicity induced by in utero DBP-exposure. The current ideas regarding the mechanisms behind phthalate induced toxicity and the recent assessment of the risk phthalates pose to the reproductive health of man and our environment are discussed in the final Chapter.

7 Final Discussion

In 2001 Skakkebaek et al hypothesised that four disorders of male reproductive health, comprising a "Testicular Dysgenesis Syndrome" (TDS), were all consequences of altered development of the testis and reproductive tract during fetal life. The disorders that encompass this syndrome are described in Chapter 1. Skakkebaek et al went on to suggest that the increase in the incidence of these clinical conditions during recent times might be associated with the ubiquitous contamination of our environment by chemicals with the potential to disrupt normal endocrine dependent development, as seen in wildlife (Skakkebaek et al., 2001). When the present studies were conceived, the causal factors behind TDS were unknown. It was considered that exposure of the pregnant mother was a route of exposure to synthetic chemicals that have become ubiquitous in the modern environment (eg DBP) that warranted further investigation.

The consequences of in utero exposure of fetal rats to DBP (a known endocrine disrupting chemical) during testis development, has been explored and the observations made are presented in experimental Chapters 3 and 6 of this thesis. Studies of DBP and its metabolite MBP on rat and human fetal testis development in vitro were carried out and are reported in experimental Chapters 4 and 5, respectively. A detailed introduction to these studies is presented in Chapter 1 with a description of the scientific approaches, materials and methods used listed in Chapter 2. This final chapter aims to bring together the achievements of the present studies in the context of relevant publications of work by other research groups.

The toxic effect of phthalates on the adult testis has been known since the 1980's but more recently effects of phthalate exposure on the developing testis have been reported. Toxicity was thought to result from disruption of the complex interactions between Sertoli, Leydig, and germ cells associated with decreased fetal androgen production (Mylchreest et al., 2002). It was logical to presume that these effects were mediated by alteration of signal molecules in the local testis environment (Li and Kim, 2003). Other evidence suggested that the balance between androgen and oestrogen action may be important in the induction of reproductive tract abnormalities that manifest as the symptoms of human TDS (Sharpe, 2003).

The present studies demonstrate that late gestational exposure to high levels of the endocrine disrupting chemical DBP significantly reduces fetal testis testosterone production and permanently alters aspects of testis pathology. The precise mechanism through which DBP induces these changes is not clear but the major finding of the present research is the demonstration that short-term (e19.5-e20.5) *in utero* exposure to 500mgDBP/kg/day can induce the same range of changes in the development of rat testes at e21.5, as seen with longer-term (e13.5-e20.5) gestational exposure, changes reported to occur in human TDS. Additionally, it has been demonstrated that the exploitation of an *in vitro* system to investigate the effects of the DBP

metabolite MBP on the development and steroidogenic regulation of rat fetal testis explants, could not mimic the effects of the DBP in vivo so this approach was concluded to be an ineffective means through which to explore the mechanisms behind the DBP-induced changes in testis development. This also meant that using the in vitro approach to investigate any effect of DBP/ MBP on human fetal testis explants resulted in limited scope for comparison of effects of phthalate exposure between the two species.

The DBP-exposure regime described in Chapter 3 was first reported as a potential animal model for human TDS in Fisher et al (2003). This paper highlighted the important role that animal models can play to enable characterisation of the normal and abnormal mechanisms of fetal testis development. The DBP-exposure regime provides a useful tool to examine the altered mechanism(s) that underlie human TDS because the testicular and other changes in DBP-exposed rats have all been reported to occur in human TDS (Fisher et al., 2003). This work became the starting point for the rest of the experiments presented in this thesis.

It was initially thought that Sertoli cell disorders (e.g. failure to mature during fetal/ adolescent development) that do not present until adulthood, may be an underlying cause of human TDS (Sharpe et al., 2003). This hypothesis was supported by the critical role Sertoli cells have in the orchestration of fetal testis development. Fisher et al (2003) demonstrated that immature Sertoli cells are present in the seminiferous tubules of adult male rats following in utero DBP exposure, at an age when these cells should have undergone terminal differentiation into their adult form (Fisher et al., 2003). However, the present data and other sources prompted a revised hypothesis in which any Sertoli cell effect observed in the DBP model is considered to be secondary to an earlier effect on Leydig cells (Chapter 3). This Leydig cell effect presents as reduced fetal testosterone production, from at least e19.5 (Chapter 3 and Mahood et al., 2005). In the present studies, the level of expression of the protein ($P450_{scc}$) that performs the rate limiting enzymatic step of the steroidogenic pathway is reduced following both long-term and short-term in utero DBP-exposure regimes. Other groups have shown significant dose-dependent reductions in mRNA and protein concentration of the cholesterol transport proteins SR-B1 and StAR, as well as the steroidogenic protein $P450_{scc}$ at e19.5 following daily exposure to DBP from e12.5, at doses of 50-500mgDBP/kg/day (Lehmann et al., 2004). Together these data demonstrate a coordinated, dose-dependent reduction in the expression of key genes and proteins involved in cholesterol transport and steroidogenesis and a corresponding reduction in testosterone in fetal testes following maternal exposure to DBP.

The effect of DBP on steroidogenesis appears to be unique to the testis in the fetal rat as there was no change in $P450_{scc}$ expression observed in adrenal glands taken from DBP exposed rat fetuses as reported in Chapter 6. Thompson et al (2005) reported no statistically significant change in the

level of adrenal corticosterone production nor any statistically significant change in the expression of genes required for steroidogenesis in the adrenal at e19.5 following daily DBP exposure from e12.5 (Thompson et al., 2005).

The in vitro models presented in Chapters 4 and 5 were unable to demonstrate any reduction in testosterone production when the cultured fetal testis explants were treated with high doses of MBP or DBP. Despite the potential of the in vitro system to enable manipulation of the effects of phthalates on fetal testis explants and to explore any parallels in sensitivity to phthalates between the rat and the human, this approach was unable to mimic the rat in vivo model. It may be this approach can make a useful contribution to other studies. It might be that MBP undergoes further metabolism outside the testis in vivo and it is this alternative metabolite that induces toxicity hence the testis explants were not affected by the high doses of MBP in vitro. Evaluation of the metabolism of a single dose of DBP in female rats confirmed the identities of DBP metabolites (MBP, monohydroxybutylphthalate and butanoic acid phthalate) in free and glucuronidated forms, in urine and in maternal and fetal plasma (Fennell et al., 2004). MBP was the major metabolite in maternal and fetal plasma and in amniotic fluid initially, but by 24h after dosing, the major metabolite in amniotic fluid was MBP-glucuronide (Fennell et al., 2004). It is possible to measure a wide range of phthalate metabolites in human urine (Silva et al., 2003). Recent improvements have enabled the separation and quantification of MBP from its structural isomer mono-iso-butyl phthalate (MiBP) (Silva et al., 2004). Further in vivo/ in vitro studies are required to reveal whether the metabolites of DBP, other than free MBP e.g. MiBP contribute to DBP induced testicular toxicity in vivo.

The levels of some phthalate metabolites in the urine of pregnant women in the USA were recently reported by Swan et al (2005). The urinary levels of four phthalate metabolites (MEP, MBP, MBzP and MiBP) were inversely related to the AnoGenital Index (AGI=AnoGenital Distance (mm)/weight at examination (kg)) of their male offspring, indicative of undermasculinisation. AGD growth is sexually dimorphic and androgen dependent. The AGI was significantly correlated with the proportion of boys that presented with incomplete testicular descent and AGD was also significantly correlated with penile volume (Swan, 2005). AGD in male rats is normally about twice that in females, and a similar sex difference is evident in humans. AGD was not assessed for the fetal rats in the present studies but has been reported to be reduced in male rats after in utero exposure with phthalates at concentrations >250mg/kg/day (Ema and Miyawaki, 2001; Foster, 2005; Zhang et al., 2004). However, this AGD must be affected by lower levels of phthalate exposure in humans because according to the Swan et al (2005) data, reduced AGI was measured in boys born to mothers with phthalate levels similar to those found in about 25% of the adult female U.S. population.

The Swan data suggests that phthalates could have similar adverse effects on fetal testosterone production in humans as they do in rats. If this interpretation is correct, it provides a strong link between phthalate exposure in humans as one potential cause of TDS disorders, a conclusion that has widespread public health implications (Sharpe, 2005). These novel human data support the hypothesis that prenatal phthalate exposure at environmental levels could adversely affect male reproductive development in humans and challenges the previous view of regulatory authorities that exposure to phthalates at levels well below effect levels in animals, poses no risk to human health (McKee et al., 2004). However, it must be noted that these correlations do not rule out other lifestyle factors that may contribute to the changes observed but were not quantified. It must be noted that there was no significant effect of MBP on the level of basal testosterone production by human fetal testis explants incubated with 10^{-3}M MBP for 48h, compared to untreated explants, as reported in Chapter 5. This suggests that 48h may not be sufficient time for the low levels of environmental phthalates to exert sufficient influence on the developing testis to induce a significant reduction in testosterone production.

As well as changes in testosterone production it is apparent that DBP has no direct effect on sexual determination but it does affect testis differentiation, as shown by the regions of focal testicular dysgenesis presented in Chapters 3 and 6. The formation of these dysgenetic areas can be illustrated schematically (Figure 7.1). The precise mechanism initiated by in utero DBP-exposure that disrupts the completion of normal testis cord formation is still unclear. The present studies have advanced our understanding of the critical window in fetal testis cord formation that DBP-exposure can induce focal dysgenesis. The long-term DBP-exposure regime (e13-20.5) produced a shift in the distribution of Leydig cells from small and dispersed clusters to larger more centralised aggregates. This pattern was also induced by the short-term DBP-exposure regime (e19-20.5) reported in Chapter 6, though to a lesser degree, corresponding to the reduced exposure duration. The relevance of this shift in distribution of the steroidogenic cells throughout the fetal testis is unclear, especially as the short-term DBP-exposure regime reported in Chapter 6 induced a more significant reduction in testicular testosterone production than did the long-term DBP-exposure regime. The differences in the impact of these treatment regimes on the physiology of the fetal rat testis warrant further investigation. The ontogeny of the effects induced by the short-term DBP-exposure regime should be followed up and compared against those described in Chapter 3 for the long-term treatment regime. Experiments reported by Carruthers and Foster (2005) were designed to identify the critical days of gestation that DBP exposure induced abnormal development of the testis. Testicular malformations were most significantly increased in DBP-exposed males only on GD 16 and 17. Together with the data presented in Chapter 6, it can be concluded that 2-day DBP exposure is highly detrimental to the developing reproductive tract

of the male fetus with the most severe abnormal development induced following exposure between e16–18.5 (Carruthers and Foster, 2005).

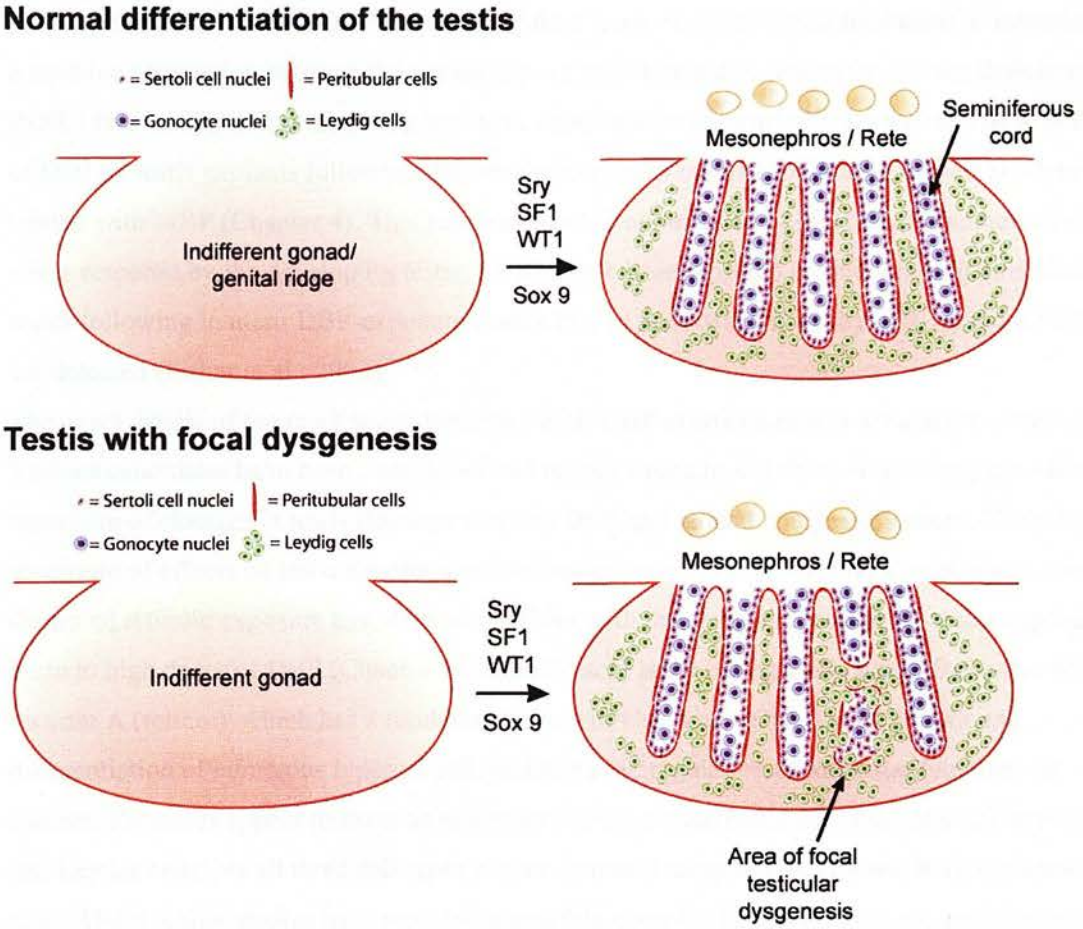

Figure 7.1 Schematic representation of normal (top panel) vs. dysgenetic (lower panel) testicular development, as hypothetically induced by in utero DBP-exposure. Reproduced from RM Sharpe, 2005.

The short-term DBP-exposure regime induced abnormal formation of gonocytes into multinuclear cells located in the centre of the testicular cords at a significantly higher incidence than the long-term regime did. The appearance of multinuclear gonocytes (MNGs) following in utero DBP exposure has been investigated and reported by Kleymenova et al (2005). Consistent with the findings of the present studies, Kleymenova et al reported that histological examination showed a low incidence of MNGs in the normal fetal rat testis whereas the *in utero* DBP-exposure regime significantly increased the number of these abnormal germ cells. As seen in the present studies, these abnormal cells were not apoptotic in the fetal testes, nor reported in the postnatal testis. The paper went on to assess the interactions between Sertoli and the MNGs and noted that Sertoli cells in DBP-exposed fetal testes had retracted apical processes, altered organization of the vimentin cytoskeleton, and abnormal cell-cell contacts with gonocytes (Kleymenova, 2005). This data was

considered to indicate that abnormal interactions between Sertoli and germ cells during fetal life play a role in the development of MNGs and is consistent with the abnormal gonocyte positioning and seminiferous cord distension reported in Chapters 3 and 6.

In vitro manipulation with DBP treatment of fetal testis explants would lend itself to investigating a possible association between the appearance of multinucleated gonocytes and the deficiency in INSL3 (see McKinnell et al, 2005), but these abnormal fetal germ cells were observed in the cords of fetal rat testis explants following 48h incubation under control conditions as well as when treated with MBP (Chapter 4). This suggests that the appearance of MNGs may be indicative of a stress response by the developing testis. MNGs have been reported as frequent in perinatal rat testes following in utero DBP-exposure from e19.5 to postnatal d4, but by d10, no more MNGs are detected (Fisher et al., 2003).

The exact details of the mechanism through which DBP exerts its effects are not yet understood. Various candidates have been considered and mostly found to fall short of inducing the wide repertoire of changes in testis development that DBP and related compounds cause. The wide repertoire of effects on testis development and testosterone production that manifest as a result of disrupted retinoid exposure has many similarities with the changes reported for testes exposed in utero to high doses of DBP (Chapter 3). Retinoic acid is the biologically active derivative of vitamin A (retinol), which has a fundamental role in vision as well as in the growth and differentiation of numerous types of cell, including the regulation of testicular functions in rodents. Retinoids appear to exert an action on the three main testicular types of cell (Sertoli, germ and Leydig cells), as all three cell types express retinoid receptors (RARs and RXRs) (Boulogne et al., 1999). Other studies have reported a possible interplay between retinoids, androgen and inhibin signalling systems in adult Sertoli cells as part of the regulation of spermatogenesis (Zhuang et al., 1997). More relevant to these studies with fetal testes was the report that retinoids play a negative role on steroidogenic activity during the differentiation of rat fetal Leydig cells (Livera et al., 2004). Studies demonstrated that endogenous retinol inhibits normal differentiation and/or function of fetal Leydig cells and may be required for the normal regression of fetal Leydig cell function that occurs after e18.5 (Livera et al., 2004).

Alternatively, peroxisome proliferators such as phthalates, are thought to act through peroxisome proliferator-activated receptors (PPAR), members of the steroid/thyroid hormone receptor superfamily. PPARs form heterodimers with RXRs and regulate the transcription of genes that contain peroxisome proliferator-responsive elements (PPREs) (Dufour et al., 2003). How phthalates might interact with PPARs is not yet known but it is proposed that any regulatory mechanism that is able to explain the testicular damage by phthalates *in vivo* should include PPARs as key players. Lapinskas et al (2005) determined that there are marked species-specific

differences in the response to peroxisome proliferators, with rodents highly susceptible and humans reportedly resistant to their short-term effects, perhaps due to lower levels of constitutive PPAR expression in the human compared to rodents (Lapinskas et al., 2005). Combined with the low levels of environmental phthalate exposure, it might be concluded that the risk of phthalate-related toxicity in humans was unlikely to be great. However, the phthalate-syndrome of effects on male reproductive tract development in the rat has parallels with the reported human testicular dysgenesis syndrome (Fisher et al., 2003). As well as disrupting the cellular level of testicular formation, the long-term in utero DBP-exposure regime reported in Chapter 3 revealed changes in testis descent and penis formation that did not manifest until later in life. These symptoms together with the testicular events of abnormal germ development (as an indicator of predisposition to testicular cancer) and reduced Sertoli cell proliferation (resulting in a restricted capacity for sperm production) are consistent with the four manifestations that comprise human TDS (Skakkebaek et al., 2001).

The present studies have contributed to our understanding of DBP (a ubiquitous contaminant of the modern environment) as an endocrine disruptor that also specifically targets the fetal testis. These experiments have helped to rule out the use of an in vitro system with fetal testis explants to elucidate the still unknown mechanism behind phthalate toxicity. The in vivo data presented is in agreement with data published by other groups. In these and other studies, DBP has been shown to effect a marked reduction in fetal testicular testosterone production during the critical window for androgen dependent development of the male reproductive tract (Mahood et al., 2005). A second Leydig cell product, Insl3, is also significantly down regulated and together with the decrease in testosterone is likely to be responsible for the cryptorchidism commonly reported in phthalate-treated animals (McKinnell et al., 2005). The testosterone decrease is mediated by changes in the expression levels of a number of enzymes and transport proteins involved in normal testosterone biosynthesis and transport in the fetal Leydig cell (Foster, 2005).

Since these studies were conceived, the biggest change in the understanding of DBP-induced toxicity has been the shift away from the idea that the Sertoli cell was the mediator of DBP toxicity, towards the view that the Leydig cell is the primary target, due to the adverse effect of DBP on steroidogenesis and Insl3.

Although no accurate cause and effect relationship has yet been demonstrated to induce TDS after in utero exposure of humans to phthalate esters, humans are exposed to and produce the critical phthalate metabolites associated with increased incidence of TDS symptoms in animal models (Swan, 2005). These metabolites have been detected frequently in the blood of the general population, with high levels in the urine of women of child bearing age as well as in children and even in human amniotic fluid (Foster, 2005). To this end, supplementary research is required to

unravel the mechanisms of phthalate toxicity on the fetal testis further and to clarify the risk these ubiquitous chemicals pose to human reproductive health.

8 Publications and References

8.1 Publications

Hallmark, N., et al. (in preparation) Effects of monobutyl- and di (*n*-butyl)- phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. *Endocrinology?*

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